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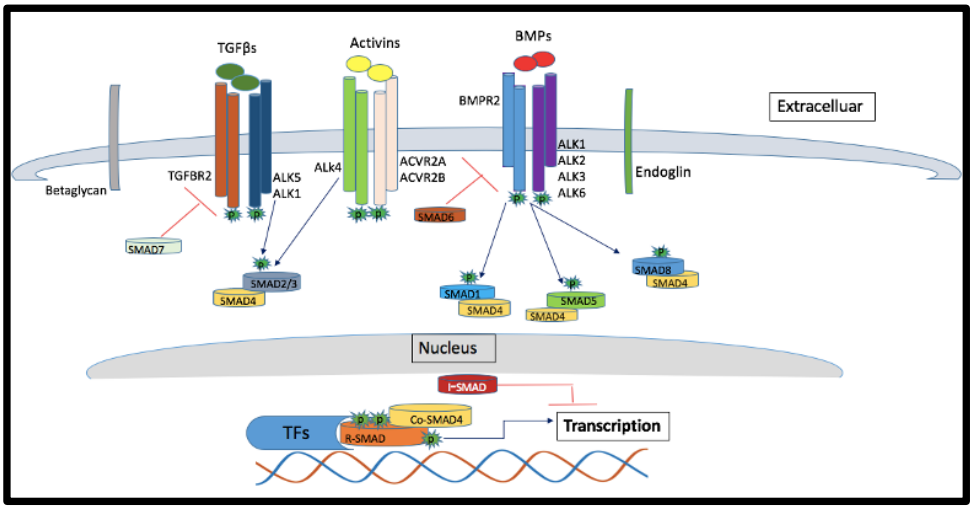
DELINEATING THE CELLULAR MECHANISMS OF ENDOPLASMIC RETICULUM-RETAINED ENDOGLIN MUTANTS CAUSING HEREDITARY HEMORRHAGIC TELANGIECTASIA TYPE 1

Nesrin Mohammed Haider Gariballa



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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

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Cover: The TGF beta SMAD- dependent signaling pathway

(Photo: By Nesrin Mohammed Haider Gariballa)

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Declaration of Original Work

I, Nesrin Mohammed Haider Gariballa, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Delineating The Cellular Mechanisms of Endoplasmic Reticulum-Retained Endoglin Mutants Causing Hereditary Hemorrhagic Telangiectasia Type 1"*, hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Bassam R. Ali, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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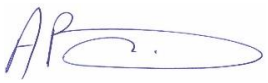
   Signature  Date 12/06/2023

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Abstract

Endoglin, also known as cluster of differentiation 105 (CD105), is an auxiliary receptor in the TGFβ signaling pathway. It is predominantly expressed in endothelial cells as a component of the heterotetrameric receptor dimers comprising type I, type II receptors and the binding ligands. Mutations in *ENG*, the gene encoding endoglin, have been associated with Hereditary Hemorrhagic Telangiectasia type 1 (HHT1), a rare autosomal dominant inherited disorder affecting about 1 in 5000-8000 individuals, which is generally characterized by vascular malformations. Secretory and many endomembrane proteins synthesized in the Endoplasmic Reticulum (ER) are subjected to a highly stringent protein folding and assembly quality control mechanisms to ensure that only properly folded and assembled proteins are transported forward through the secretory pathway to their final destinations. We have previously demonstrated that some endoglin variants causing HHT1 are retained in the ER and failed to traffic to their normal localization at the plasma membrane, which suggested the possible involvement of the ER Associated protein Degradation (ERAD) in their molecular pathology. In this study, we have investigated the detailed degradation routes of endoglin wild type and two ER-retained mutant variants, P165L and V105D. Stably transfected HEK293 cells expressing Wild Type (WT) and the two mutants were treated with proteasomal and lysosomal inhibitors to elucidate their cellular degradation pathways and the molecular mechanisms underlying the loss of function phenotype associated with the disease-causing variants. Our results show that WT endoglin has a relatively short half-life of less than 2 hours and degrades through both the lysosomal and proteasomal pathways, whereas the two disease-causing mutant variants (P165L and V105D) are relatively stable with half-life of more than 16 hours and predominantly degrade through the proteasomal ubiquitin pathway. Furthermore, we have demonstrated that endoglin variants P165L and V105D are significantly accumulated in the CRISPR-Cas9-generated HEK293 cells deficient in HRD1 E3 ubiquitin ligase; a major ERAD component. Therefore, our results conclusively confirm the involvement of ERAD in the cellular mechanisms of some HHT1 disease-causing missense variants. These findings might

pave the way for more in-depth research studies that could open new windows for future therapeutic interventions for patients with HHT1.

We have also investigated if ER-retained HHT1-causing endoglin variants L32R, C53R, V105D, I271N, P165L and C363Y would exert dominant negative effects by hijacking the wild type allele in the ER. Our results show that these ER- retained endoglin mutant variants are able to form heterodimers with the wild type protein. Crucially, they exhibit considerable dominant negative effects manifested in significant entrapment of wild type endoglin within the ER leading to reduced maturation. This suggest that in addition to haploinsufficiency due to mutations in one allele, heterozygous carriers of these ER-retained variants are likely to lose part of the function of the WT allele. These findings may help explain some of the phenotypic heterogeneity amongst HTT1 patients.

Keywords: Endoglin, TGFβ signaling pathway, Hereditary hemorrhagic telangiectasia type 1, ERAD, Endoplasmic reticulum, ER-retained mutant variants.

Title and Abstract (in Arabic)

تحديد الأليات الخلوية للإندوجلين الطافر المحتجز في الشبكة الاندوبلازمية والمسبب لداء توسع الشعيرات النزفي الوراثي

الملخص

الإندوجلين، والذي يطلق عليه أيضاً معقد التمايز 105 (CD105)، هو أحد المستقبلات المساعدة في ممر الإشارات TGFβ. يتم التعبير عنه غالباً في الخلايا البطانية كواحد من مكونات المستقبلات الثنائية المكونة للمعقدات الرباعية غير المتجانسة من النمط الأول والثاني والروابط الحصرية .اتضح ارتباط الطفرات في الجين المسؤول عن تكوين الإندوجلين، والذي يطلق عليه *ENG*، بالإصابة بداء توسع الشعيرات النزفي من النمط الأول HHT1، وهو مرض وراثي نادر ينتقل بصفة جسدية سائدة. يترافق هذا المرض عموماً بتشوهات وعائية. تتعرض العديد من البروتينات المصنعة في الشبكة الاندوبلازمية ER، سواء المفرزة منها أو المرتبطة بالغشاء، إلى مراحل ضبط جودة صارمة لآليات طي البروتينات وتجميعها. يهدف ذلك إلى التأكد من أن البروتينات المطوية والمجمعة بشكل صحيح هي وحدها من ستتقدم في السبل الافرازية باتجاه وجهتها النهائية. اكتشفنا مسبقاً أن بعض الطفرات المسببة لداء توسع الشعيرات النزفي من النمط الأول HHT1 تؤدي إلى استبقاء البروتين واحتجازه في الشبكة الاندوبلازمية وفشله في الانتقال عبر الخلية ليصل إلى موضعه الصحيح على الغشاء الخلوي. ويقترح هذا تورطاً محتملاً لآلية الانحلال البروتيني المرتبط بالشبكة الاندوبلازمية، والتي يطلق عليها اختصاراً ERAD، بالآلية الامراضية الجزيئية لهذا الداء. يستكشف هذا البحث بالتفصيل، ولأول مرة، سبل انحلال الاندوجلين الطبيعي إضافة إلى نوعين طافرين منه يحملان الطفرة P165L وD105V. لتحقيق هذا الهدف، استخدمت خلايا HEK293 المعالجة بحيث تعبر عن النمط الطبيعي من الاندوجلين والنمطين الطافرين منه. تمت معالجة هذه الخلايا بمثبطات البرتيوزوم والليزوزوم من أجل الكشف عن سبل الانحلال الخلوي والآليات الجزيئية التي تقف خلف فقدان الوظيفة الذي تتسم به الطفرات المسببة للمرض. أظهرت النتائج أن الاندوجلين الطبيعي لديه معدل نصف حياة قصير نسبياً يبلغ أقل من ساعتين حيث ينحل عن طريق كل من السبيل الليزوزومي والبوتيوزومي. بالمقابل، فإن الطفرتين المسببتين للمرض تسببتا بثباتية عالية للبروتين، ويحث الانحلال بها غالباً عبر السبيل البروتيوزومي. ومن ثم، اتضح أن الاندوجلين الحامل لأي من الطفرتين يتجمع بشكل واضح في خلايا HEK293 التي ينقصها ليغاز اليبوكويتين HRD1 E3، والذي يعد مكوناً رئيسياً في آلية ERAD. هذه النتائج تؤكد بوضوح أن آلية ERAD تلعب دوراً في الآليات الخلوية لبعض الطفرات المسببة لداء توسع الشعيرات النزفي من النمط الأول. يمكن لهذه النتائج أن تمهد الطريق لبحوث أعمق قد تتيح علاجات لهذ المرض في المستقبل.

استقصى هذا البحث أيضا ما إذا كانت طفرات الاندوجلين المحتجزة للبروتين في الشبكة الاندوبلازمية (وهي كل من طفرة P165L، V105D، L32R، C53R، I271N، C363Y) أن تتسبب بتأثر سلبي سائد على الأليل الطبيعي. أظهرت النتائج أن الإندوجلين الطافر قادر على تكوين معقدات ثنائية غير متجانسة مع البروتين الطبيعي. كما

أظهرت الطفرات المختلفة تأثيرات سلبية متباينة أدت إلى احتجاز جزئي للإندوجلين الطبيعي في الشبكة الاندوبلازمية. يمكن لهذه النتائج أن تفسر تنوع مظاهر المرض بين مرضى داء توسع الشعيرات النزفي من النمط الأول.

مفاهيم البحث الرئيسية: الاندوجلين، الشبكة الاندوبلازمية، توسع الشعيرات النزفي الوراثي، معقد التمايز، الطفرة، مكونات المستقبلات الثنائية؛ الخلايا البطانية.

Author's Contribution

The contribution of Nesrin Gariballa to the dissertation was as follows:

1. Participated in the planning of the experiments under prof. Bassam Ali's supervision.

2. Took sole responsibility of the experimental work including data collection, evaluation and analysis.

3. Participated as a first author in the publications generated from data presented in this thesis.

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I express my love and sincere gratitude to my friends who have always been there for me in both the good and bad times, supporting, helping and encouraging me throughout.

I would not have been able to go through the demands and challenges of my PhD journey without the support of my husband Salah, my children and, above all, my beloved mother. To those I love I dedicate my PhD thesis.

Dedication

I dedicated this thesis to my parents Faiza and Mohammed-Nour, my husband Salah and my children Mohammed, Samar, Muaz and Ammar.

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List of Abbreviations

4-PBA	4-Sodium Phenylbutirate
ACVR2A	Activin A Receptor Type 2A
ACVR2B	Activin A Receptor Type 2B
ACVRL2	Activin A Receptor Like Type 2
AD	Alzheimer Diseases
ALK1	Activin Receptor-Like Kinase Type 1 Receptor
AMHR2	Anti -Müllerian Hormone Receptor Type 2
APP	Amyloid Precursor Protein
AVMs	Arteriovenous Malformations
BMP	Bone Morphogenetic Proteins
BMPR2	Bone Morphogenetic Protein Receptor
CF	Cystic Fibrosis
CNX/CRT	Calnexin and Calreticulin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DTT	Dithiothreitol
EC	Endothelial Cells
ECD	Ecto-Domain
ECL	Enhanced Chemiluminescence Plus Reagent
EDEM	ER Degradation-Enhancing α-Mannosidase-like Protein)
EerI	Eeyarestatin I
EMT	Epithelial–Mesenchymal Transition
ENG	Endoglin
ER	Endoplasmic Reticulum
ER	Endoplasmic Reticulum
ERAD	ER- Associated Protein Degradation
ERALD	ER-to-Lysosomes-Associated Degradation
ERK/MAPK	Kinase/Mitogen-Activated Protein Kinase
ERQC	ER Quality Control
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FPAH	Familial Pulmonary Arterial Hypertension
FZ-CRD	Frizzled Cysteine-Rich Domain
GCD2	Granular Corneal Dystrophy Type 2
GDF2	Growth Differentiation Factor
GFD	Growth Differentiation Factors
GI	Gastrointestinal
GLU I	Glucosidases I
GLUII	Glucosidases

GP78	Glycoprotein 78
HD	Huntington Disease
HEK293	Human Embryonic Kidney 293
HHT	Hereditary Hemorrhagic Telangiectasia
HRD1	3-Hydroxy-3-Methylglutaryl Reductase Degradation
JNK	c-Jun N-Terminal Kinase
Kif	Kifunensine
KO	Knockout
LDLR	Low Density Lipid Receptor
MKKs	MAP Kinase Kinases
mTORC2	Mammalian Target of Rapamycin Complex 2
OR-C	C-Terminal Orphan Domain
OR-N	N-Terminal Orphan Domain
PARK2	Parkin2
PBS	Phosphate-Buffered Saline
PD	Parkinson Diseases
PIK3	Phosphatidylinositol-3-Kinase
polyQ	Polyglutamine
PTC	Premature Termination Codon
RFP	Red Fluorescence Protein
S/Thr	Serine/Threonine
SAHA	Suberolyanilide Hydroxamic Acid
SDS/PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
T/TKD	Threonine Cytoplasmic Kinase Domain
TAK1	TGF-β-Activated Kinase 1
TBS-T	Tris-Buffered Saline-Triton
Telo-HAEC	Telomerase-Human Aortic Endothelial Cells
TGFβ	Transforming Growth Factor Beta
TMD	Trans-Membrane Domain
TUDCA	Tauroursodeoxycholic Acid
UPR	Unfolded Protein Response
VLDLR	Very Low Density Lipid Receptor
WT	Wild Type
ZP	Zona Pellucida Domain

Chapter 1: Introduction

1.1 Overview

The Endoplasmic Reticulum Quality Control mechanism (ERQC) is a surveillance and protein quality control mechanism which ensures that, for newly synthesized proteins to proceed to the next compartment in the secretory pathway, they must pass a predetermined quality criteria that define the "normal". The problem is encountered when proteins persistently fail to reach their native folding and assembled states due to disease-causing mutations or other abnormality (Tax et al., 2019). In addition, some aberrant, but partially functional proteins can be prematurely selected for degradation and disposal by the ERQC mechanism, which can lead to their loss of function within cells.

The ER has adopted a highly sophisticated and conserved mechanism known as ER-Associated Protein Degradation (ERAD), to discard improperly folded proteins and unassembled orphaned subunits of multi-subunit complexes. This is a complex process that involves the coordination of many proteins in both the ER and the cytoplasm with input from the nucleus through the Unfolded Protein Response (UPR) (Christianson & Ye, 2014). In order for misfolded proteins to be disposed of, they need to be recognized, retrotranslocated into the cytosol, polyubiquitinated and then extracted from the ER membrane and consequently degraded in the cytosol by proteasomes (Wu & Rapoport, 2018). The ERAD quality control mechanism has been involved in the pathogenesis of numerous human genetic conditions including cystic fibrosis, emphysema, Robinow syndrome, Alzheimer Diseases (AD), Parkinson disease and other neuro degenerative diseases (Kaneko et al., 2017). This is simply because almost a third of all the cellular proteins are targeted to the ER in transit to their final destinations and are therefore subjected to this highly stringent quality control system (Sun & Brodsky, 2019). We have shown in previous studies a possible involvement of the ERAD mechanism in the pathogenesis of monogenic diseases such as autosomal dominant Hereditary Hemorrhagic Telangiectasia type1 and 2 (HHT1, HHT2) and familial Pulmonary Arterial Hypertension (FPAH), that are caused by mutations in *ENG*, *ACVRL1* and *BMPR2*, respectively (Ali et al., 2011; Hume et al., 2013; John et al., 2015). The mutant

proteins (endoglin, Alk1 and BMPR2) encoded by the three genes (*ENG*, *ACVRL1* and *BMPR2*), respectively, are transmembrane receptors in the TGFβ signaling superfamily. The focus of this project is the elucidation of the molecular mechanisms involved in pathogenesis of HHT1 caused by missense mutations in endoglin; a type III co-receptor in a TGFβ signaling pathway. Endoglin expressed in the blood vessels plays a key role in the control of endothelial cells proliferation, migration and capillary formation (Bofarid et al., 2021). Consequently, HHT1 is characterized by vascular malformation which can manifest in variable symptoms that ranges from spontaneous nasal bleeds (epistaxis) and mucocutaneous telangiectases to more severe phenotypes in the form of Arteriovenous Malformations (AVMs) in the brain, lung or liver (Faughnan et al., 2020). Involvement of the ERAD mechanism in the pathogenesis of HHT1 may open a window for potential new therapeutic targets which involves the manipulation of the ER quality control mechanism to enhance mutant protein folding and trafficking. Up to now, limited number of studies have been carried out to investigate the cellular and molecular mechanisms of HHT1. The aim of this study is to further elucidate the detailed cellular mechanisms of a number of missense endoglin mutations causing HHT1.

1.2 Statement of the Problem

Hereditary Hemorrhagic Telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, is an autosomal dominant inherited disease with an estimated prevalence of 1:5,000 -1:8,000 individuals depending on the population investigated (Bofarid et al., 2021; Dakeishi et al., 2002). The disease significantly affects the quality of life of patients and could be life threatening in severe cases (Westermann et al., 2003). Furthermore, the heterogeneity of the disease phenotype amongst patients carrying the same mutations makes it difficult for clinicians to make an early diagnosis. So far, no definitive cure has been proposed for HHT and treatments focus mainly on symptoms managements. Although, geneticists have worked extensively on the discovery of genes associated with HHT, nonetheless only few have focused on investigating the detailed cellular pathways through which disease-causing variants exert their pathological consequences. It is essential to investigate how a mutation can alter a protein's biosynthesis, trafficking and degradation in a series of processes governed by stringent cellular and molecular quality control mechanisms. Our group have suggested the

possible implication of ERAD in the degradation of some ER-retained endoglin HHT1-causing variants (Ali et al., 2011). Thus, in this thesis, I set out to further elucidate the proposed cellular mechanisms of some of the ER-retained endoglin variants. We also hypothesized that some ER-retained endoglin mutants might bind and trap the wild type protein in the ER and I therefore investigated the possibility of a dominant negative effect exerted by some of these variants. This dominant negative effect is expected to have a compounding effect towards endoglin's loss of function leading to disease phenotype. In conclusion, our aim is to shed light on the cellular mechanisms of HHT1 that can be used as a platform for the development of potential therapeutic interventions.

1.3 Research Objectives

1. Investigation of the cellular half-life and the degradation route(s) of endoglin Wild-Type (WT) and some ER-retained mutant variants.
    - In order to achieve this objective, stably transfected HEK293 cells expressing WT and mutant variants of endoglin were used as a cellular model for our investigations. Assessment of mutant proteins stability and cellular half-life was determined using cycloheximide chase assay and Western blotting. Cycloheximide is a translation inhibitor used on cells *in vitro* to determine a protein's half-life over a course of a 24 hours experimental timeframe. Stably transfected cells were then individually treated with proteasomal, lysosomal and ERAD inhibitors in order to identify the pathway through which endoglin WT and mutant variants are being degraded. Accumulation of the protein was used an indication on the involvement of the inhibited degradation pathway.

2. Analysis of the stability of the mutant variants in HEK293 cell lines deficient in HRD1 E3 ubiquitin ligase, a major component of ERAD retro-translocon channel.
    - CRISPR-Cas9 gene editing system was used to generate bi-allelic HRD1-Knockout HEK293 cell lines. The stability of endoglin WT and mutants were assessed in the generated HRD1-KO clones. The increased stability and accumulation of the endoglin mutant variants in the HRD1-KO cells further demonstrates the involvement of ERAD in their degradation.

3. Explore the potential for manipulating the folding and trafficking of endoglin mutants using chemical chaperones.
    - We have utilized the general chemical chaperones glycerol and Sodium Phenylbutirate (4-PBA) to investigate a possible rescue effect on the ER-retained mutant variants P165L and V105D. HEK293 cells stably expressing WT endoglin and the two variants were incubated with 5% glycerol or 2.5 mM of 4-PBA.
    - In order to investigate the localization of mutant variants in treated cells, live cells were collected and immunostained with endoglin conformational specific antibody (P3D1), as described in the methods section. The antibody can only recognize plasma membrane localized endoglin. A FITC conjugated secondary antibody was used to visualize immunostained endoglin protein using fluorescence associated cell sorting (FACS).

4. Investigate the possibility of a dominant negative effects exerted by ER-retained endoglin variants on the WT allele.
    - In order to examine if mutant variants trapped in the ER heterodimerize with WT endoglin and thus hindering its normal cellular trafficking to the plasma membrane, HA-tagged immunoprecipitated endoglin mutant variants were run on SDS-PAGE gels and probed with antibodies against a specific Myc-tagged WT endoglin. In addition, confocal microscopy imaging was used to evaluate the change in the subcellular localization of the WT protein when co-expressed with ER-retained mutants.

1.4 Relevant Literature Review

1.4.1 The TGFβ Superfamily Components

The Transforming Growth Factor Beta (TGFβ) signaling pathway plays key roles in diverse cellular activities starting during embryogenesis and continue throughout

adulthood. That includes cellular growth, differentiation, immunological responses and apoptosis. Consequently, dysfunctions in components of this pathway have been implicated directly or indirectly in various human diseases (Gariballa & Ali, 2020; Zi, 2019). The various genome projects have revealed that mammalian genomes encode 33 TGFβ-related polypeptides that include three TGFβ isoforms, Bone Morphogenetic Proteins (BMPs), Growth Differentiation Factors (GFDs), nodals and inhibins (Morikawa et al., 2016). These ligands transduce the signaling cascade through transmembrane receptors and intracellular transducer proteins referred to as SMADs. Upon initiation of the signaling pathway via binding of the ligand to serine/threonine type II receptor, activation of the type I receptor causes dimerization of the type II and type I receptors in a heterotetramic complex (Figure 1). Both types of receptors have a cytoplasmic kinase domain, however type II receptor kinase domain is constitutively active, which facilitates prompt activation of the cytoplasmic tail of the type I receptor upon ligand binding. Phosphorylated type I receptor activates SMADs transducers and the signal is propagated to the nucleus through a cascade of phosphorylation reactions carried out by the SMAD transcription factors (Groppe et al., 2008). The formation of the heterotetrameric receptor complex is ligand dependent as well as context specific (Hata & Chen, 2016). For example Activins, nodal and subsets of MBPs share their Type I and Type II receptors, on the other hand, TGFβ isoforms exclusively keep their receptors to themselves (Groppe et al., 2008).

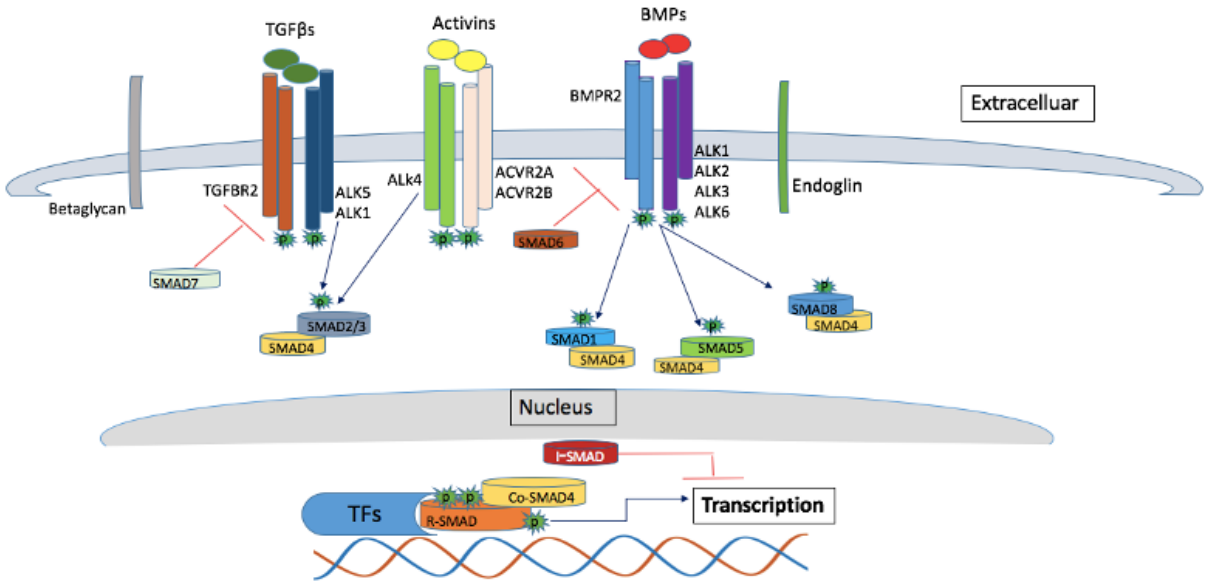

Figure 1: The TGF beta SMAD- dependent signaling pathway

The diagram shows some of the major components of the TGFβ signaling pathway. SMAD- dependents signal transduction is initiated with the binding of the ligands (e.g. TGFβ, BMP, activins etc.) to the serine/threonine type II receptor that phosphorylates and activates the type I receptor which causes dimerization of the type II and type I receptors in a heterotetrameric complex. The signal is then propagated to the nucleus through phosphorylation of SMAD transcription factors. The image represents only few of the ligands/receptors/SMADs possible signal transduction combinations (Gariballa & Ali, 2020).

The human genome encodes five type II receptors: Activin A Receptor Type 2A (ACVR2A), Activin A Receptor Type 2B (ACVR2B), Bone Morphogenetic Protein Receptor (BMPR2), Activin A receptor like Type 2 (ACVRL2) and Anti-Müllerian Hormone Receptor (AMHR2) and seven type I receptors (Activin Receptor-Like Kinase 1-7; ALK1-7) (Hata & Chen, 2016).

Structurally, type I and type II receptors show very close similarities, as they both include a disulfide rich ectodomain (120 residues), a single-spanning transmembrane domain (30 residues) and a cytoplasmic Serine/Threonine (Ser/Thr) kinase domain (400 residues) (Figure 2) (Hinck, 2012). However, type I receptor has distinctive region of 30 conserved glycine/Serine region located upstream of the kinase domain. This region is targeted by type II receptors for phosphorylation, a mechanism that drives the activation of the type I receptor (Weiss & Attisano, 2013).

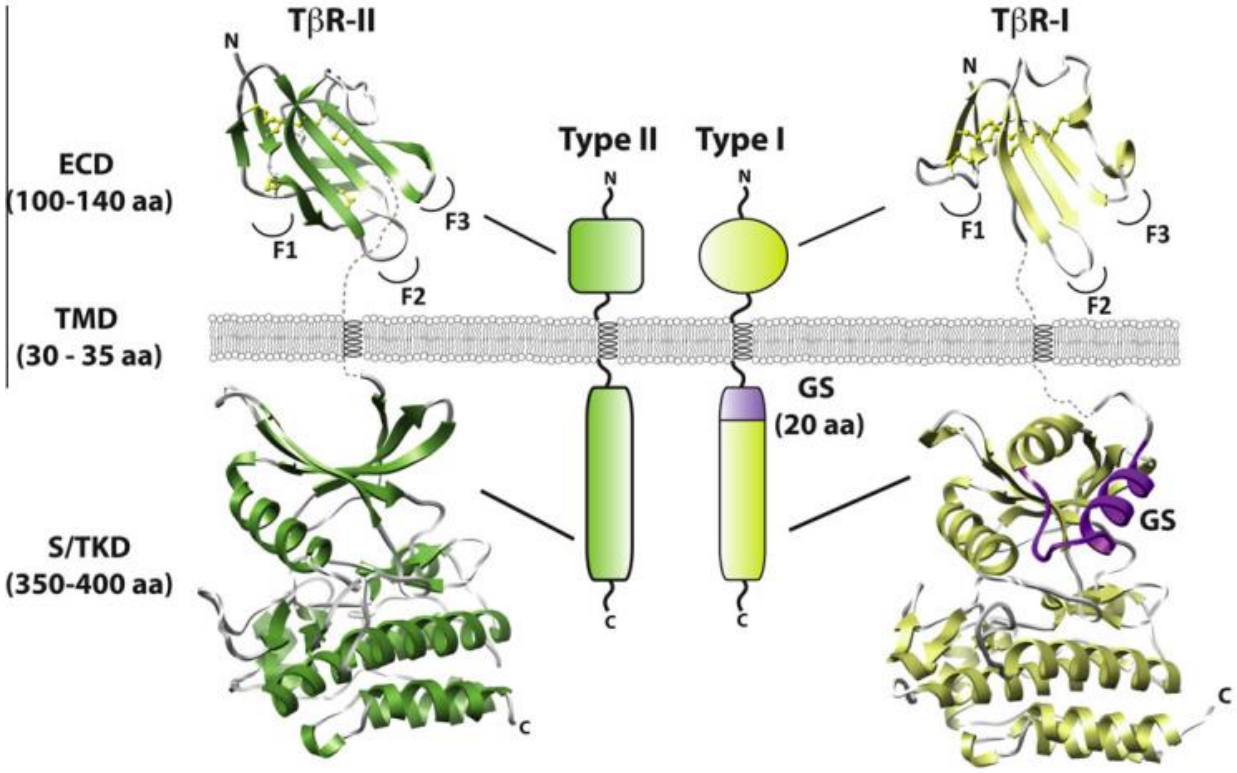

Figure 2: TGFβ Type I and type II receptors domains

The diagram shows as an illustration of the three structurally similar domains shared by both types of receptors Ecto-Domain (ECD), Trans-Membrane Domain (TMD) and serine/ threonine cytoplasmic kinase domain (T/TKD). It shows type II receptor's distinct glycine-serine rich regulatory domain, known as the GS box (GS). The crystal structures of the domains were obtained from PDB database. Image adapted from (Hinck, 2012).

TGFβ type III receptors (sometimes referred to as co-receptors) include Betaglycans and endoglin, which both have no kinase activity. However, they can bind a wide range of TGFβ ligands with high affinity and facilitates ligand binding to TGFβ type II receptors and hence enhance ligand-receptor complex binding and augment its downstream signaling effect (Vander Ark et al., 2018). The availability of the TGFβ type III receptors have added an extra layer of complexity to the regulation of the TGFβ superfamily. TGFβ type III receptors are comprised of an extracellular domain, a transmembrane domain and a short cytoplasmic tail. While the extracellular domain facilitates the presentations of the ligand to signaling receptors, cleavage of this domain yields a soluble form of the co-receptor that can promote regulation of the signaling cascade through binding and sequestering the signaling ligand (Weiss & Attisano, 2013).

SMADs are considered as the signal transducer in the TGFβ signaling pathway. They propagate the signal from cell membrane to the nucleus in a context dependence manner. Up to date, eight SMAD proteins have been identified in humans that have been classified into three classes, Receptor mediated SMADs (R-SMADs), Common partner SMADs (Co-SMADs) and Inhibitory SMADs (I-SMADs) (de Caestecker, 2004). R-SMADs include SMAD2 and SMAD3 that are activated in response to binding of Activins and TGFβ proteins to the TGFBR2 and ACVR1B receptors. On the other hand, R- SMADs such as SMAD1, SMAD5 and SMAD8 are phosphorylated by BMPR2 receptor in response to the binding of BMP proteins. Activation of the second class SMADs (SMAD4-Co SMAD) is receptor independent, however their function is crucial for the receptor regulated SMADs. The third class of SMADs (SMAD6 and SMAD7) function as antagonists that inhibit the signaling of R-SMADs and Co- SMADs by competing with the ligands that trigger receptor phosphorylation. SMAD6 generally inhibits BMP activation, while SMAD7 generally work as a feedback regulator for TGFβ activation (Pérez-Gómez et al., 2010) (Moustakas & Heldin, 2009) (Hata & Chen, 2016). Beside the canonical SMAD regulated signaling pathway, TGFβ ligands can also regulate cellular physiological responses through non–SMAD signaling proteins, which have actually preceded the discovery of SMADs (Moustakas & Heldin, 2005). Non-SMAD signaling proteins downstream of the TGFβ receptors can attenuate and regulate the signaling pathways in various modes of actions. They can directly interact with type I receptors and become phosphorylated without a direct interaction with SMADs. On the other hand, non-SMAD proteins can transiently interact with SMADs in order to facilitate the activation of signaling pathways such as Extracellular Signal Regulated Kinase/ Mitogen-Activated Protein Kinase (ERK/MAPK) pathways, Rho-like GTPase signaling pathways, and Phosphatidylinositol-3-Kinase (PI3K)/ AKT pathways (Zhang, 2009). ERK/MAP kinase pathway is activated via tyrosine phosphorylation of cytoplasmic signaling protein; ShcA, by activated TGFβ type I receptor, followed by the formation of ShcA/Grb2/Sos complex and subsequent activation of Ras GTPase, Raf, MEK and ERK1/2 kinases. ERK1/2 can phosphorylate transcription factors as well as SMADs and hence regulate gene expression (Tzavlaki & Moustakas, 2020). The activation of the MAPK pathway is predominantly observed in epithelial cells triggered

by a variety of cell growth stimuli such as insulin, thrombin, epidermal and hematopoietic growth factors (Hartsough & Mulder, 1995). c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways can also be activated independent of SMAD through the activation of MAP Kinase Kinases (MKKs). Both JNK and p38 MAP kinase pathways play key roles in a variety of cellular functions such as differentiation, apoptosis and inflammation (Yu et al., 2002). MAP3K7, also known as TGF-β-Activated Kinase 1 (TAK1), is a well-known activator of p38 MAP kinase pathway downstream of TGFβ ligands that can also phosphorylate R-SMADs at the linker region promoting a negative feedback regulation of the canonical TGFβ signaling pathway (Tzavlaki & Moustakas, 2020).

Phosphoinositide 3-Kinases (PIK3) exhibit constitutive interaction with type II receptors through its p85 regulatory unit, while interaction with the type I receptors occurs upon the TGFβ binding to the receptor complex, which leads to the activation of the PIK3/AKT signaling pathway (Yi et al., 2005). This pathway promotes cellular survival and growth in response to extracellular signals in multiple cellular processes including glucose metabolism, apoptosis and cell proliferation. Activation of PIK3 pathway can also activate Mammalian Target of Rapamycin Complex 2 (mTORC2), leading to the phosphorylation of AKT, which collectively contribute to Epithelial–Mesenchymal Transition (EMT) and cell migration (Lamouille et al., 2012).

TGFβ ligands can also induce the Rho GTPases signaling pathway independent of SMADs regulation. Activation of RhoA and Cdc42 GTPases in epithelial cells play key roles in cytoskeleton regulation and cell motility (Edlund et al., 2002). This pathway can also be negatively regulated by Par6, a negative regulator of epithelial cells polarity that is closely associated with TGFβ type I receptor. Par6 phosphorylation facilitates the recruitment of ubiquitin ligases that labels RhoA GTPase for degradation (Ozdamar et al., 2005).

In addition to the SMAD and non-SMAD regulators of TGFβ signaling pathways, it is very important to note that cross-talk between the TGFβ signaling pathway and other pathways can also occur (Luo, 2017). The activation as well as the function of the various components of the TGFβ pathways are constantly regulated by various signaling

pathways that control cellular processes, adding to the complexity and diversity of its functions.

1.4.2 Involvement of TGFβ Signaling Pathway Components in Human Single Gene Disorders

As illustrated in the previous section, TGFβ signaling pathway plays key roles during the early embryonic developmental stages, in which axis formation and tissue specifications are determined (Harradine & Akhurst, 2006). Therefore, germline mutations in the TGFβ pathway components have given rise to a heterogeneous spectrum of hereditary diseases with phenotypes mainly associated with malformations in the cardiovascular, muscular and skeletal system. I have conducted an extensive literature and databases searches to document the involvement of mutations in the TGFβ pathway components in the development of monogenic hereditary diseases. This exercise revealed 47 monogenic diseases associated with genetic mutations in 24 out of 41 TGFβ components (Table 1). The majority of the diseases are autosomal dominant with variable penetrance and expressivity. Similar phenotypes can also arise from mutations affecting related genes in the signaling pathway such as Hereditary Hemorrhagic Telangiectasia Type 1 and 2 (HHT1, HHT2), which are caused by mutations in *ENG* and *ACVRL1*, respectively. In addition, mutations in *GDF2* encoding the BMP9 ligand, which is considered the main binding ligand for both endoglin and ALK1 in endothelial cells, has been associated with HHT type 5 (HTT5) (Bailly et al., 2010; Balachandar et al., 2022). A study by Saito et al. (2017) has elegantly demonstrated, through crystal structure analysis, how homodimeric BMP9 interacts with orphan domains of endoglin. In this interaction, the Zona-pellucida domain of endoglin mediates homodimerization, which, together with the orphan domains, establishes a specific spatial arrangement that securely clamps the BMP9 ligand between homodimeric endoglin (Saito et al., 2017) . In addition, BMP9 ligand was shown to interact specifically with the hydrophobic residues of Orphan domain 2 (OR2, 47-199 residues) without direct interaction with the orphan domain 1 (OR1, 200-330 residues). Therefore, mutant variants within the vicinity of such interaction are predicted to affect ligand binding and may lead to pathogenic consequences.

Table 1: Monogenic diseases associated with SMAD-regulated TGFβ pathway components

***Gene/* Protein**	**Monogenic Disease/OMIM Reference**	**Reference**	**Possible ERAD Pathology**
*ACVRL1/*Alk1	1. Hereditary hemorrhagic telangiectasia syndrome 2 (HHT2; 600376) (AD)	(Johnson et al., 1996).	Yes (Hume et al., 2013)
ACVR1/ Activin A receptor type 1 Alk2	1. Fibrodysplasia ossificans progressiva (FOP; 135100) (A/D)	(Shore et al., 2006)	NA
TGFBR1/ TGF beta receptor type1 Alk5	1. Loeys-Dietz syndrome 1 (LDS1; 609192). 2.Multiple self-healing squamous epithelioma (MSSE; 132800)	(Loeys et al., 2005) (Ferguson-Smith et al., 1971)	NA
BMPR1A/ Bone Morphogenetic Protein Receptor Type 1A Alk3	1. Juvenile polyposis syndrome (JPS; 174900) (A/D). 2. Polyposis syndrome, mixed hereditary 2 (HMPS2; 610069)(A/D)	(Howe et al., 2001) (Cao et al., 2006)	NA
BMPR1B/ Bone Morphogenetic Protein Receptor Type 1B Alk6	1. Acromesomelic dysplasia, Demirhan type (AMDD; 609441) (A/R). 2.Brachydactyly A2 (BDA2; 112600)(A/D) 3. Brachydactyly A1, D (BDA1D ; 616849) (A/D)	(Demirhan et al., 2005) (Lehmann et al., 2003) (Racacho et al., 2015)	NA
TGFBR2/ Transforming growth factor beta receptor 2	1. Loeys-Dietz syndrome 2 (LDS2; 610168). 2. Hereditary non-polyposis colorectal cancer 6 (HNPCC6; 614331)) (AD)	(Boileau et al., 1993) (Markowitz et al., 1995)	NA
BMPR2/ Bone Morphogenetic Protein Receptor Type 2	1. Pulmonary hypertension, primary, 1 (PPH1;17860).(AD) 2. Pulmonary venoocclusive disease 1; (PVOD1;265450) (AD)	(Lane et al., 2000) (Machado et al., 2001)	Yes (John et al., 2015)
ACVR2B/ Activin A receptor type 2B	1. Visceral heterotaxia4 (HTX4; 613751)(AR).	(Kosaki et al., 1999)	NA
AMHR2/ Anti-Mullerian Hormone Receptor Type 2	1. Persistent Muellerian duct syndrome type 2 (PMDS2; 261550)(AR)	(Imbeaud et al., 1995)	NA

Table 1: Monogenic diseases associated with SMAD-regulated TGFβ pathway components (Continued)

***Gene/* Protein**	**Monogenic Disease/OMIM Reference**	**Reference**	**Possible ERAD Pathology**
ENG/ endoglin	1. Hereditary hemorrhagic telangiectasia syndrome 1 (HHT1 ; 187300)(AD)	(McAllister et al., 1994)	Yes (Gariballa et al., 2022) (Ali et al., 2011)
BMP1/ Bone Morphogenetic Protein 1	1.Osteogenesis imperfecta 13 (OI13; 614856)	(Martínez-Glez et al., 2012)	NA
BMP2/ Bone Morphogenetic Protein 2	1. Brachydactyly A2 (BDA2 ;112600))(A/D) 2. Short stature, facial dysmorphism, and skeletal anomalies with or without cardiac anomalies (SSFSC; 617877) (A/D)	(Dathe et al., 2009) (Tan et al., 2017)	NA
BMP4/ Bone Morphogenetic Protein 4	1. Microphthalmia, Syndromic 6 (A/D) (MCOPS6; 607932) 2. orofacial cleft 11 (OFC11; 600625)	(Bakrania et al., 2008) (Suzuki et al., 2009)	NA
GDF2/ BMP9 Morphogenetic Protein9/Growth Differentiation Factor 2	1.Hereditary hemorrhagic telangiectasia type5 (HHT5; 615506) (AD)	(Wang et al., 2016)	NA
GDF1/ Growth Differentiation Factor 1	1. Congenital heart defects, multiple types, 6 (CHTD6; 613854) (AD). 2. Right atrial isomerism (RAI; 208530)	(Karkera et al., 2007) (Eronen et al., 2004)	NA
GDF3/ Growth Differentiation Factor 3	1. klippel-feil syndrome 3 (KFS3 ; 613702) (AD). 2. Microphthalmia, isolated, 7 (MCOP7; 613704) (AD). 3. Microphthalmia, isolated, with Coloboma 6(MCOPCB6; 613703)(AD).	(Ye et al., 2010)	NA

Table 1: Monogenic diseases associated with SMAD-regulated TGFβ pathway components (Continued)

***Gene/* Protein**	**Monogenic Disease/OMIM Reference**	**Reference**	**Possible ERAD Pathology**
GDF5/ Growth Differentiation Factor 5	1. Acromesomelic chondrodysplasia Hunter-Thomson type (AMDH;201250)(AR). 2. Acromesomelic chondrodysplasia, Grebe type (AMDG; 200700)(AR) 3. Brachydactyly C (BDC;113100))(AD &AR) 4. Du Pan syndrome ((DUPANS; 228900)) (AR) 5. Symphalangism, proximal 1B (SYM1B; 615298) 6. Multiple synostoses syndrome 2 (SYNS2 ; 610017). 7. Brachydactyly A2 (BDA2; 112600).	(Faiyaz-Ul-Haque et al., 2002) Polinkovsky et al., 1997) (Thomas et al., 1997) (Seemann et al., 2005) (Plöger et al., 2008) (Miyamoto et al., 2007) (Byrnes et al., 2010)	NA
TGFb1/ Transforming Growth Factor Beta 1	1. Camurati-Engelmann disease (CAEND; 131300) 2. Inflammatory bowel disease, immunodeficiency and encephalopathy (IBDIMDE; 618213)	(Kinoshita et al., 2000) (Kotlarz et al., 2018)	NA
TGFb2/ Transforming Growth Factor Beta2	1. Loeys-dietz syndrome 4 LDS4; 614816 (AD)	(Lindsay et al., 2012)	NA
TGFb3/ Transforming Growth Factor Beta	1. Loeys-dietz syndrome 4 (LDS5; 615582) (AD) 2. Arrhythmogenic right ventricular dysplasia-1 (ARVD1; 107970)	(Rienhoff et al., 2013) (Beffagna et al., 2005; Rampazzo et al., 2003)	NA
SMAD3/ SMAD Family Member	1. Loeys-dietz syndrome 3 (LDS3; 613795) (AD)	(van de Laar et al., 2011)	NA
SMAD4/ SMAD Family Member	1. Juvenile polyposis syndrome/hereditary hemorrhagic telangiectasia syndrome (JPS; 174900)(AD) 2. (JP/HHT) (JPHT, 175050) (AD), 3. Myhre syndrome (MYHRS; 139210)	(Burger et al., 2002) (Howe et al., 1998) (Le Goff et al., 2011)	NA
SMAD6/ SMAD Family Member	1. Aortic valve disease (AOVD2; 614823) 2.Craniosynostosis (CRS7; 617439) 3. Radioulnar synostosis (RUS; 179300)	(Tan et al., 2012) (Timberlake et al., 2016) (Yang et al., 2019)	NA

Table 1: Monogenic diseases associated with SMAD-regulated TGFβ pathway components (Continued)

***Gene/* Protein**	**Monogenic Disease/OMIM Reference**	**Reference**	**Possible ERAD Pathology**
SMAD8/9/ SMAD Family Member	1. Primary pulmonary hypertension 2 (PPH2; 615342)	(Shintani et al., 2009)	NA

The Table lists all SMAD-regulated components of the TGFβ signaling pathway and associated monogenic diseases. Components with no identified disease association so far are ACVR1B, ACVR1C, ACVR2A, TGFBR3, INHBA, Inhibin subunits A, B, BB & C, BMP 3, 5, 6, 7 & 10, GDF7, SMAD 1, 2, 5 & 7. Abbreviations: AR; Autosomal recessive, AD; Autosomal dominant, NA; Not available

1.4.3 Hereditary Hemorrhagic Telangiectasia Type 1(HHT1)

HHT1, also known as Rendu-Osler-Weber syndrome, is an autosomal dominant inherited disease with an estimated prevalence of 1:5,000 -1:8,000 individuals depending on the population investigated (Bofarid et al., 2021; Dakeishi et al., 2002). A much higher prevalence of 1:2351 has been reported in France arising from a haplotype inherited from inhabitants of the Haut-Jura Mountains more than three centuries ago (Bideau et al., 1980; Lesca et al., 2008). However, the highest prevalence rate of 1:1131 was reported amongst the Afro-Caribbean population of the Netherlands Antilles (Westermann et al., 2003).

Up to date, there are four different types of HHT disorders, which are all associated with components of the TGFβ signaling pathway (Table 2) (Figure 3) (Bofarid et al., 2021). However, *ENG* and *ACVRL1* remain the most common genes associated with HHT with ~80% of HHT patients were found to have mutations in either *ENG* or *ACVRL1*. On the other hand, only 2% of the patients have mutation in *SMAD4* associated with Juvenile polyposis HHT (JHHT). HHT5 have also been associated with mutations in *GDF2* gene expressing BMP9 ligand, that interacts with Alk1 and endoglin in the same signaling pathway and causes a phenotype similar to HHT (Ruiz-Llorente et al., 2017). Overall, all types of HHT share a common feature of vascular dysplasia, however, they differ in the frequency and location of the vascular lesions.

Table 2: HHT classification according to genetic association

HHT Type	**Gene Associated**	**Location**	**Protein**	**Phenotype**
HHT1	*ENG*	9q34.1	Endoglin Type III receptor	Pulmonary and cerebral AVMs, mucocutaneous telangiectasia, and epistaxis
HHT2	*ACVRL1*	12q13.13	ALK1 Type I receptor	Higher prevalence of hepatic AVMs compared to HHT type 1
HHT5	*GDF2*	10q11.22	BMP9 Bone Morphogenetic Protein 9	HHT type phenotype
JPH Juvenile polyposis and HHT	*SMAD4*	18q21.2	SMAD4 signaling transcription factor	Combination of juvenile polyposis and HHT

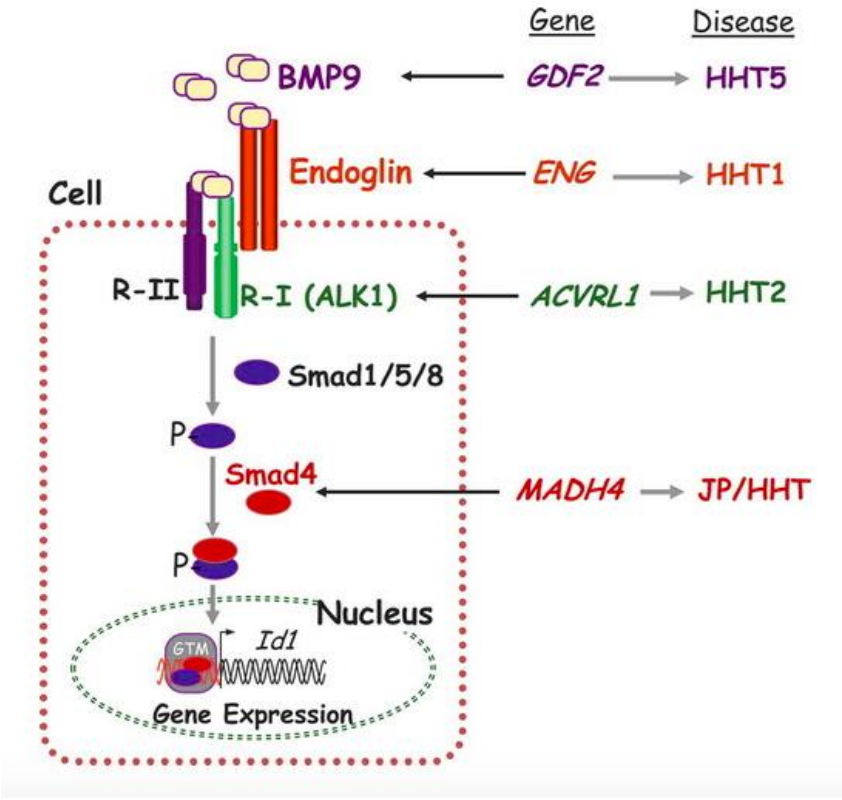

Figure 3: TGFβ pathway components associated with HHT

The diagram describes the signaling cascade triggered by the binding of BMP9 ligand to the heterotetrameric complex of Type II receptor, phosphorylated Alk1 receptor and endoglin. The signal is propagated to the nucleus through phosphorylated SMAD 1/2/3 and SMAD4 (Co-SMAD). Mutations in *ENG*, *ACVRL1*, *MADH4* and *GDF2* expressing endoglin, Alk1, SMAD4 and BMP9 are associated with HHT1, HHT2, JP/HHT and HHT5, respectively (Ruiz-Llorente et al., 2017)

Vascular malformations can manifest in variable symptoms that range from spontaneous nasal bleeds (epistaxis) and mucocutaneous telangiectases to more severe phenotypes in a form of AVMs in the brain, lung or liver (Faughnan et al., 2020) (Figure 4). AVMs arise from the lack of intervening capillary between veins and arteries which leads to their direct connection. On the other hand, telangiectases arise from dilated and enlarged post capillary venules. Most HHT patients present with age-dependent symptoms which vary in severity; however, the proper diagnosis is often not reached till adolescence (Bernabeu et al., 2020; Faughnan et al., 2020). Around the age of 12 years, 90% of patients present with recurrent and spontaneous nasal bleed that arise from fragile

telangiectases in the mucous membrane that ruptures upon slight trauma (Ruiz-Llorente et al., 2017). By the age of 50 years, 25% of HTT patients can develop severe bleeding symptom in the Gastrointestinal (GI) tracts (Ruiz-Llorente et al., 2017). Development of large AVMs in the brain, lungs or liver are usually sudden and are often associated with bad prognosis (Gallardo-Vara et al., 2019). The heterogeneity of the disease phenotype is mainly attributed to the gene affected, mutation type and environmental triggers an individual is exposed to (Ruiz-Llorente et al., 2017).

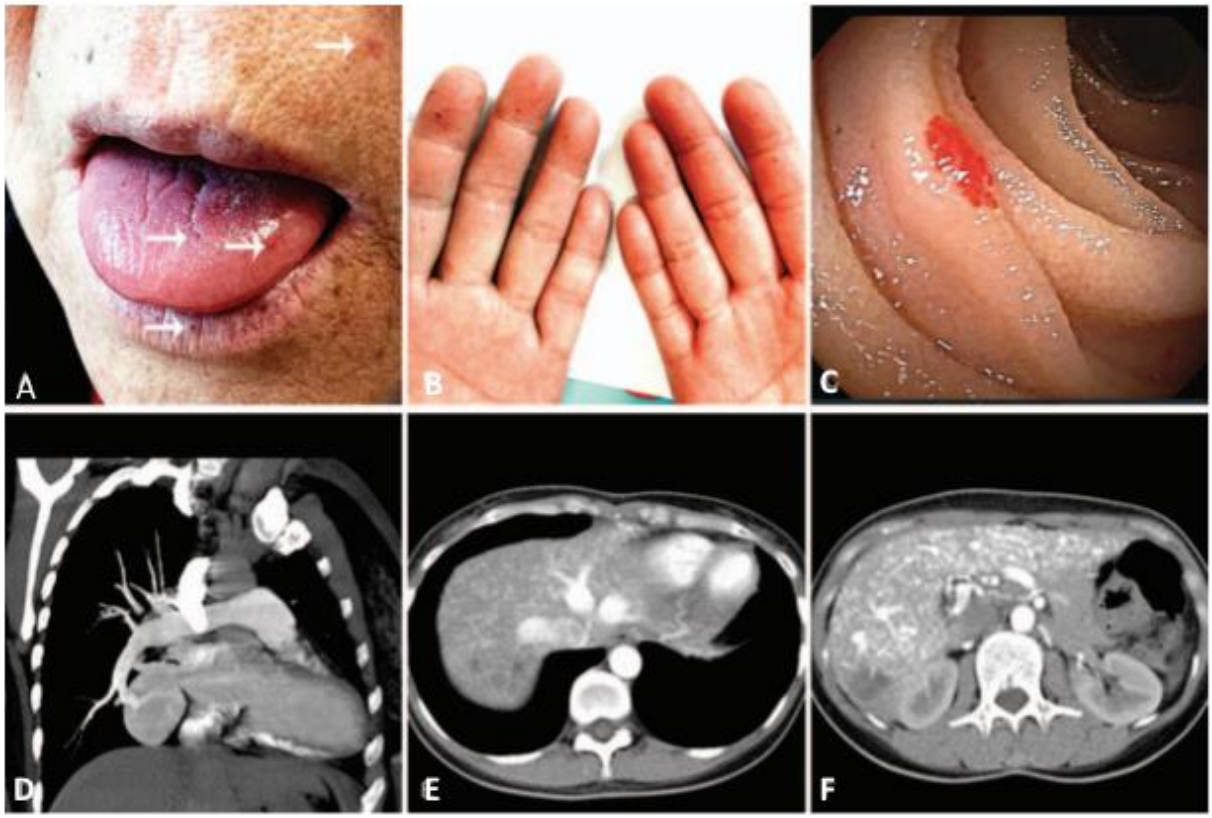

Figure 4: Clinical presentation of patients with HHT1

(A) multiple telangiectases on the face, lips and tongue. (B) Telangiectases on the hands and fingertips. (C & D) An image of CT pulmonary angiogram showing AVMs in the right lung. (E & F) CT scan images of a patient with hepatic AVMs. Images adapted from (Li et al., 2018)

Diagnosis of HHT is done through genetic testing of the associated genes or by referral to The Curaçao Diagnostic Criteria for HHT (Shovlin et al., 2000); (McAllister et al., 1994)

- Frequent nasal bleeds which can range from mild to severe.
- Telangiectases on multiple sites such as lips, nose, fingers and oral cavity.
- AVMs or telangiectases in one or more of the internal organs (lung, brain, liver, intestines, stomach and spinal cord).
- Family history.

HHT diagnosis is confirmed when at least three are present. However, a recent study has shown that the genetic diagnostic rate for patients with one to two criteria is similar to those with three criteria (Anderson et al., 2022). Furthermore it has shown that quarter of the patients with PAVMs and genetically confirmed HHT exhibit few, if any HHT features. These findings alerted medical professionals to adopt genetic testing as the most efficient diagnostic tool for HHT even if the diagnostic criteria are not met.

Up to now, there is no cure for any type of HHT, but there are several therapies used for management of the various symptoms associated with the disease. Treatment options for HHT are individualized and based on the type of vascular malformation presented (Shovlin et al., 2022).

1.4.4 Endoglin Structure

Endoglin is a type 1 membrane glycoprotein expressed at the plasma membrane as a ~180 KDa disulphide linked homodimer. Its structure is characterized by a large extracellular domain followed by a hydrophobic segment spanning the plasma membrane and a short cytoplasmic tail (Saito et al., 2017). The extracellular region is composed of an N-terminal Orphan domain (OR) and a C-terminal Zona Pellucida (ZP) domain, which is sub-classified to ZP-N terminus and ZP-C terminus (Figure 4). The extracellular domain contains ligands binding sites as well as attachment sites for *O*- and *N*- linked glycosylation (Pérez-Gómez et al., 2010; Saito et al., 2017). In contrast to the type I and type II receptors, the endoglin cytoplasmic tail does not have a kinase activity, however this domain contains Serine (Ser) and Threonine (Thr) residues at a PDZ motif, which make endoglin a substrate for TGF receptors' phosphorylation, a process that regulates interaction with these receptors (Koleva et al., 2006; Meurer & Weiskirchen, 2020).

Human endoglin exists in two alternatively spliced isoforms, a regular form (L-endoglin) and a shorter form (S-endoglin) with a shortened C terminal that lacks the phosphorylation sites and the PDZ motif, which results in functional differences compared to L-endoglin (Figure 5) (Pérez-Gómez et al., 2010). Despite the fact that S-endoglin is significantly expressed in tissues such as lung and liver, the L-endoglin isoform remains the most predominantly expressed in most tissue types, especially in the endothelium (Pérez-Gómez et al., 2010).

Structurally, endoglin belongs to the ZP family of proteins that has a characteristic stretch of 260 amino acids at the C terminus of the extracellular domain (Saito et al., 2017). In contrast to other members of the ZP family, whose ZP domain contains eight cysteine residues involved in four disulphide bridges, human endoglin contains only three disulphide bridges as a result of interaction of three pairs of cysteine residues: Cys363/Cys442 and Cys394/Cys412 in the ZP-N domain, and Cys493/Cys549 in the ZP-C domain (Ruiz-Llorente et al., 2019; Ruiz-Llorente et al., 2017). The ZP domain plays a key role in endoglin homo-dimerization (Kaneko et al., 2017).

In order for proteins to elicit a physiological response, they interact with a diverse spectrum of other proteins and biomolecules, which result in a complex network of protein-protein interaction termed (Interactome) (Bogan & Thorn, 1998). As in endoglin, homodimerization facilitates the structural arrangement and interaction with the binding ligand and the TGFβ receptors involved in signaling pathway (Saito et al., 2017). In general, the formation of homo, hetero and oligomers can be classified to stable or unstable depending on the life time of the complex, affinity between proteins and the composition of the complex (Singh & Jois, 2018). In signaling pathways interaction between proteins is an example of transient association, as proteins interact to elicit a particular physiological response and then dissociate and most likely degrade. On the other hand, homodimeric proteins like endoglin they carry out their function as a homodimer from biogenesis till degradation and hence interaction is strong and stable (Saito et al., 2017).

One of the most powerful tools employed in biochemical research to study protein-protein interactions is the Co-Immunoprecipitation assay (Co-IP). It relies on the specific

binding between a target protein and its interacting partners, enabling the isolation and subsequent analysis of the protein complex. By selectively isolating protein complexes through antibody-mediated capture, this technique offers insights into the composition, dynamics, and functional relevance of protein interaction networks. There are several biochemical assays used to study protein-protein interactions including pull-down assays, Bioluminescence Resonance Energy Transfer (BRET) and crosslinking assays.

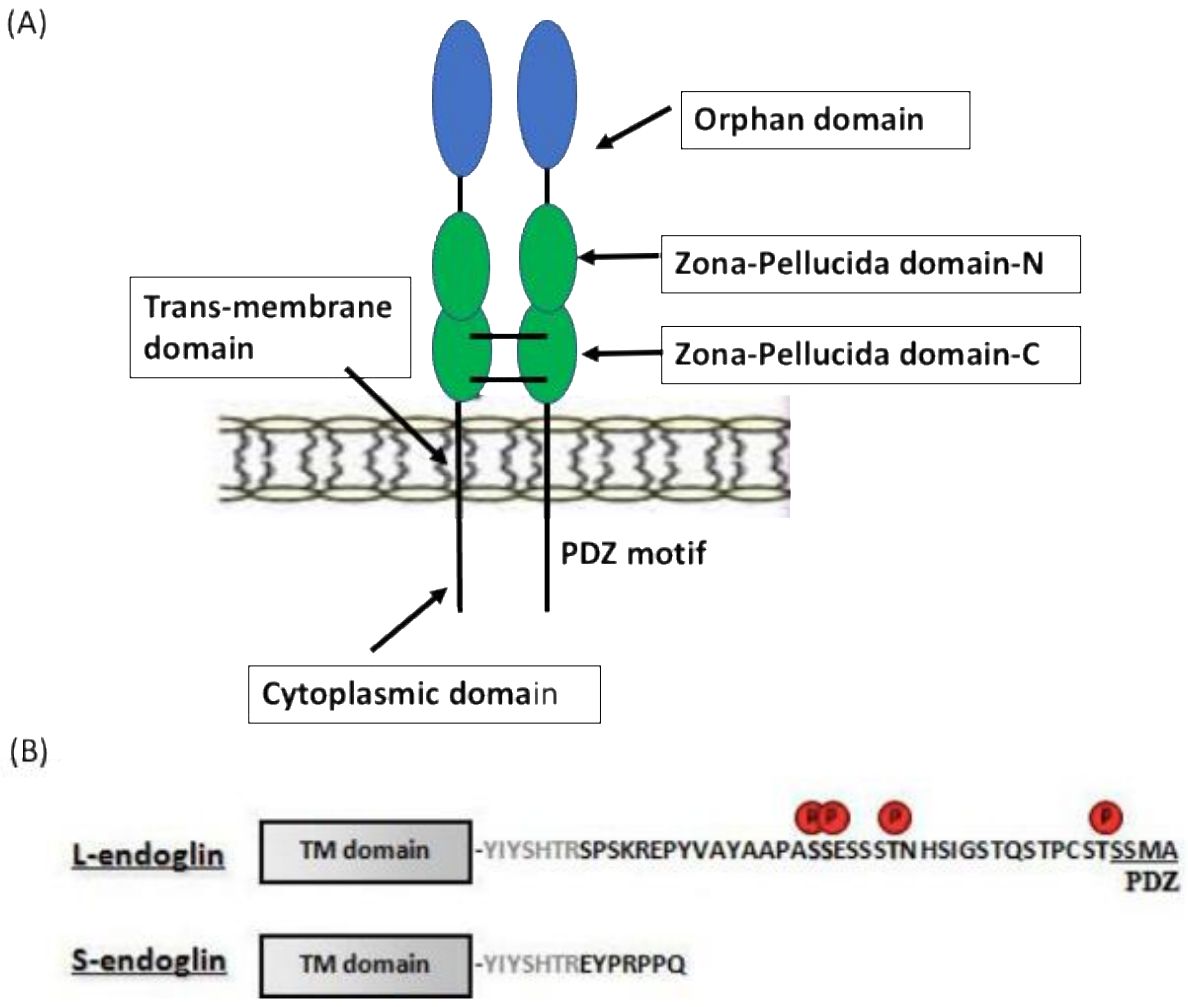

Figure 5: Schematic domain structure of human endoglin

(A)Endoglin consists of an extracellular domain comprises the orphan domain (blue), Two Zona pellucida domain (ZP-N) and (ZPC) (green), a transmembrane domain and short cytoplasmic tail. (B) The two endoglin isoforms are: Long L-endoglin and short S-endoglin. Adapted from (Pérez-Gómez et al., 2010)

1.4.5 Endoglin Physiological Functions

Endoglin expressed in the blood vessels plays a key role in the control of cell proliferation, migration and capillary formation. It can bind a range of TGFβ ligands (TGF-β1, TGF-β3, activin-A, BMP-2, and BMP-7) in the presence of signaling type I and type II receptors. It can also bind BMP9 ligand in the absence of the signaling receptors. It was also observed that endoglin interacts with various types of type I and type II receptors either through the extracellular domain or the cytoplasmic domain (Table 3). Interestingly, it was shown that endoglin is a direct substrate for the type I receptor (Alk1) in endothelial cells, which emphasizes the role of endoglin in Alk1-dependent endothelial cells growth and adhesion (Koleva et al., 2006). Alk5 transduce the signaling cascade through SMAD 2/3, while Alk1 signaling is mediated through SMAD 1/5/8. In endothelial cells, endoglin can interact with both Alk1 and Alk5 independent of the TGFβ ligand. In addition, endoglin was found to have an activation effect on Alk1/SMAD1 signaling and an inhibitory effect on the Alk5/SMAD3 pathway, which add to the complexity and diversity of its effect on TGFβ signaling pathway (Blanco et al., 2005)

Table 3: TGFβ binding proteins to endoglin

Endoglin Domain	**TGFβ Ligands**	**Type I Receptor**	**Type II Receptors**
Extra cellular domain	TGFβ1 TGFβ3 BMP2 BMP7 BMP9	Alk1 Alk2 Alk3 Alk5 Alk6	TGFβRII ActRII BMPRII
Cytoplasmic domain		Alk1 Alk5	

Adapted from (Pérez-Gómez et al., 2010)

The development of healthy vasculature is of key importance to the health of every single cell in our body. Endothelial Cells (EC) that line the inside of all blood vessels play pivotal roles in delivering oxygen and nutrients to cells, regulate immune cells trafficking and controls blood flow. The three main types of EC are venous, arterial and capillary cells. Venous EC are the main source of angiogenic expansion. They migrate upstream and against the blood flow and differentiate into tip cells that directs the arterial sprouting vessel towards the tissues (Figure 6) (Lee et al., 2022). Several studies have actually demonstrated the essential role of endoglin for EC specification and migration to prevent AVM formation (Drapé et al., 2022).

Vascular endothelial dysfunction has been associated with a wide range of hereditary and non- hereditary diseases such as ischemic heart diseases and stroke, the top causes of mortality around the world. Although vasculogenesis is established during embryogenesis, formation of new blood vessels for growing tissues and during wound healing will continue all through an individual's life. The importance of the topic has lead researchers around the world to work extensively on endothelial cells differentiation and vascular angiogenesis. The topic is also greatly implicated in cancer biology and received much attention with that regard as well (Trimm & Red-Horse, 2023).

As discussed in the previous section, HHT is characterized by the development of fragile connections between the veins and arteries which are known as AVM. It is still unknown how the decreased level of either Alk1 Type I receptor or endoglin leads to this phenotype. It has been observed that healthy endothelial cells migrate against the blood flow, a phenomenon that is not yet been fully understood. It has also been shown that inhibited expression of either endoglin or Alk1 reverse endothelial cells migration pattern. Hence, it has been suggested that Alk1-dependent signaling pathway may be contributing to the mechanism of EC migration during angiogenesis (Rochon et al., 2016; Trimm & Red-Horse, 2023).

In proliferative EC, endoglin is upregulated by Alk1 receptor and high concentration of endoglin stimulates the expression of BMP9 ligand. Paradoxically, endoglin actually exert an inhibitory effect on the TGFβ signaling pathway via the Alk5/SMAD 2/3 pathway, which suggest a regulatory role in the signaling pathway (Scharpfenecker et al.,

2007). Therefore, it appears that endoglin function is dependent on many factors including ligand concentration and cellular context (Bofarid et al., 2021).

While mutations in the *ENG* are primarily associated with HHT1, endoglin has also been implicated in other diseases and pathological conditions. Endoglin expression is often upregulated in various types of cancer, including breast, colorectal, lung, and pancreatic cancers (Balma et al., 2023). Targeting endoglin signaling is being explored as a potential therapeutic approach in cancer treatment (Li et al., 2022). Endoglin has also been implicated in preeclampsia, a pregnancy-related disorder characterized by high blood pressure and organ damage. Endoglin levels have been found to be altered in preeclampsia, and it is thought to contribute to impaired placental development and dysfunctional angiogenesis in this condition (Bokuda & Ichihara, 2023). Endoglin has been implicated in fibrotic disorders such as liver fibrosis, pulmonary fibrosis, and renal fibrosis. It plays a role in regulating the fibrotic response by influencing the activation and function of fibroblasts and the deposition of extracellular matrix (Schoonderwoerd et al., 2020). Research indicates that endoglin may play a role in controlling the uptake and metabolism of low-density lipoprotein (LDL). Endoglin has been observed to interact with LDL receptors, influencing the process of LDL receptor-mediated endocytosis and impacting the internalization of LDL particles into cells (Vicen et al., 2019). Moreover, several gene polymorphisms in endoglin have been associated with high levels of LDL-cholesterol and an increased risk of cardiovascular events. These polymorphisms along with other parameters and biomarkers were suggested to enhance the diagnosis and prognosis of susceptibility to cardiovascular damage (Garzon-Martinez et al., 2020).

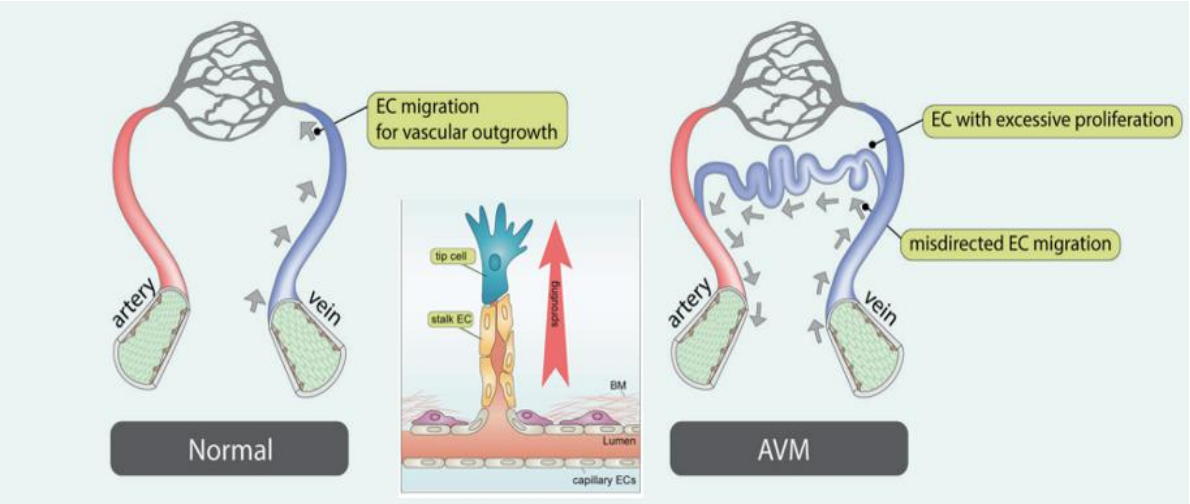

Figure 6: Traditional model for angiogenesis

Polarized normal ECs migrate against the blood flow towards the tissues for vascular formation. Disrupted EC migration causes disorientation and aberrant vascular formation that eventually lead to AVM (Lee et al., 2022).

1.4.6 ENG Mutations Causing HHT1 and their Possible Mechanisms

Up to date, over 600 mutations spanning the human endoglin have been reported (http://www.arup.utah.edu/database/ENG/ENG_welcome.php). The mutations are evenly scattered around the 15 exons of *ENG*. Missense/ nonsense mutations represent the majority of the mutations identified (Figure 7). However, no specific dominant pathogenic variant has been reported. Missense mutations result in the substitution of a single amino acid in the endoglin protein. They can affect various aspects of protein structure and function, including protein stability, folding, and ligand binding. Many of the missense mutations in *ENG* are located in the extracellular domain of endoglin, which is responsible for ligand binding (Abdalla & Letarte, 2006).

A significant percentage of mutations that cause HHT result in the creation of stop codons. This can occur either through direct nonsense mutations or as a consequence of deletions and/or insertions that disrupt the reading frame of the gene (Govani et al., 2013). Based on current molecular understanding, it is believed that most endoglin mutations that result in Premature Termination Codons (PTCs) are unable to produce an RNA molecule that can be translated into truncated proteins. In the past, it was commonly believed, supported by experimental evidence of functional changes or

dominant negative effects, that mRNAs containing premature termination codons (PTCs) could be translated into shortened proteins (McAllister et al., 1995).

A puzzling aspect within the HHT community has been the lack of identified mutations in the last coding exon. The reason for the absence of frameshift mutations that would result in stop codons in exons 13 and 14 is very likely attributed to the fact that there are no alternative reading frame stop codons present in those exons, as PTC requires a specific nucleotide substitution (Govani et al., 2013)

Transcripts from intronic regions of the *ENG* locus , have been detected in some endothelial-specific libraries (Govani et al., 2013). The finding is not surprising as it has become evident that a significant portion of eukaryotic intronic and intergenic regions in the genome are transcribed. Some of them exhibit remarkable stability, conservation, or regulation under different conditions, suggesting potential functional roles in gene regulation (Kapranov et al., 2002). The identification of such transcripts within intronic regions of the *ENG* locus, which is deleted in HHT1 disease-causing mutations, highlights additional changes in gene expression resulting from these mutations involving multiexon deletions, insertions, or rearrangements in the *ENG* gene in HHT1, which may contribute to phenotypic variability amongst affected individuals.

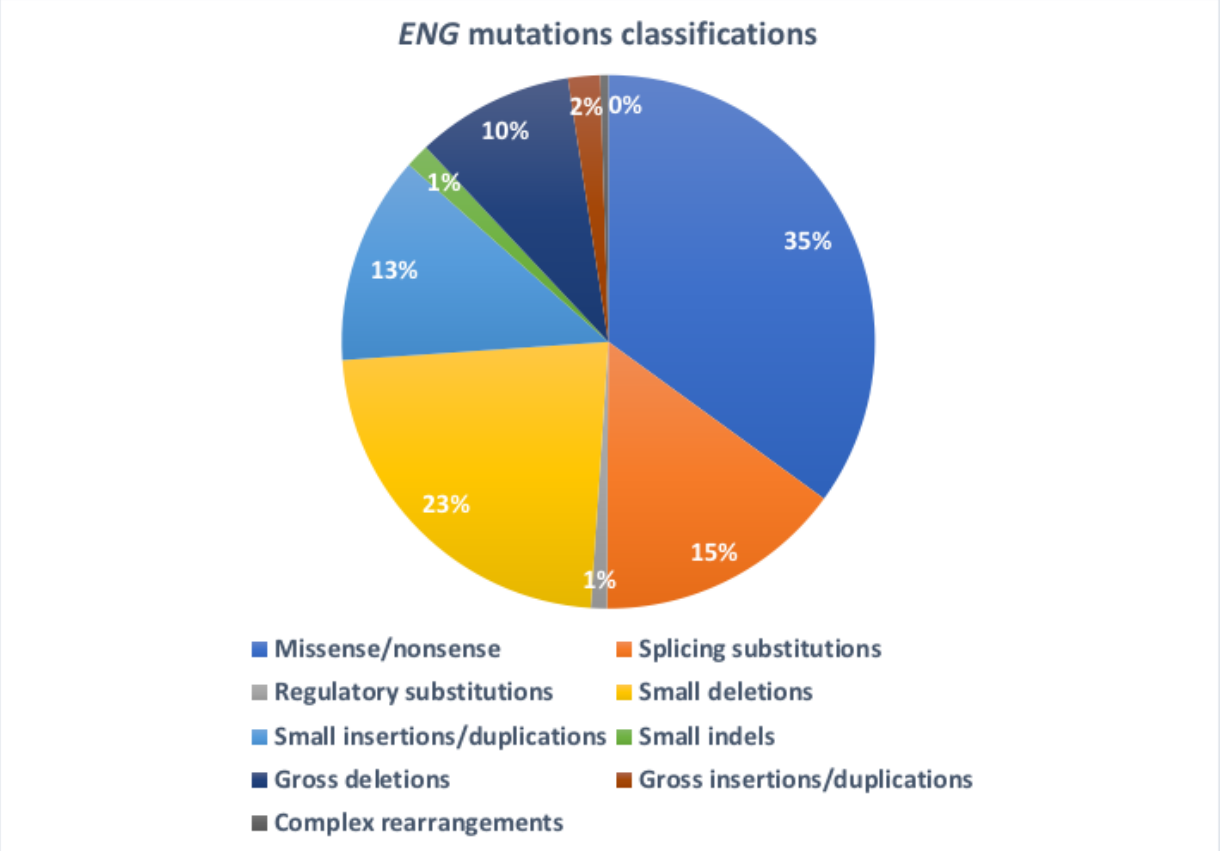

Figure 7: Classes of endoglin disease-causing mutations

Data extracted from the Human Genome Mutation Database (HGMD).

Our group has previously investigated the subcellular trafficking of 25 endoglin missense mutant variants (Ali et al., 2011). Ten mutants were found to be largely ER-retained, 8 in the orphan domain (L32R, V49F, C53R, V105D, A160D, P165L, I271N and A308D) and 2 in the ZP domain (C363Y and C382W). The rest showed normal subcellular trafficking to the plasma membrane similar to the WT. Hence, we proposed the possible implication of the ER quality control mechanism in the loss of function and degradation of these ER-retained mutant variants. Therefore, in this thesis, I set out to further investigate these findings initially by generating stably transfected HEK293 cells using plasmid vectors carrying two ER-retained variants P165L and V105D and the wild type protein. In the second part of the project, I investigated the possible dominant negative effects exerted by 6 ER-retained mutant variants P165L, V105D, L32R, C53R, I271N and C363Y (Figure 8) on the WT protein. The main reason behind this hypothesis is that endoglin dimerizes in the ER and then the dimers presumably traffic to the plasma membrane and therefore it is plausible that WT and mutant proteins also form

"heterodimers". The WT in this case might get trapped due to its association with the ER-retained mutants. In addition, it has been observed that the clinical presentations of patients, who are normally heterozygous, are variable indicating possible different levels of loss of function or involvement of modifier genes.

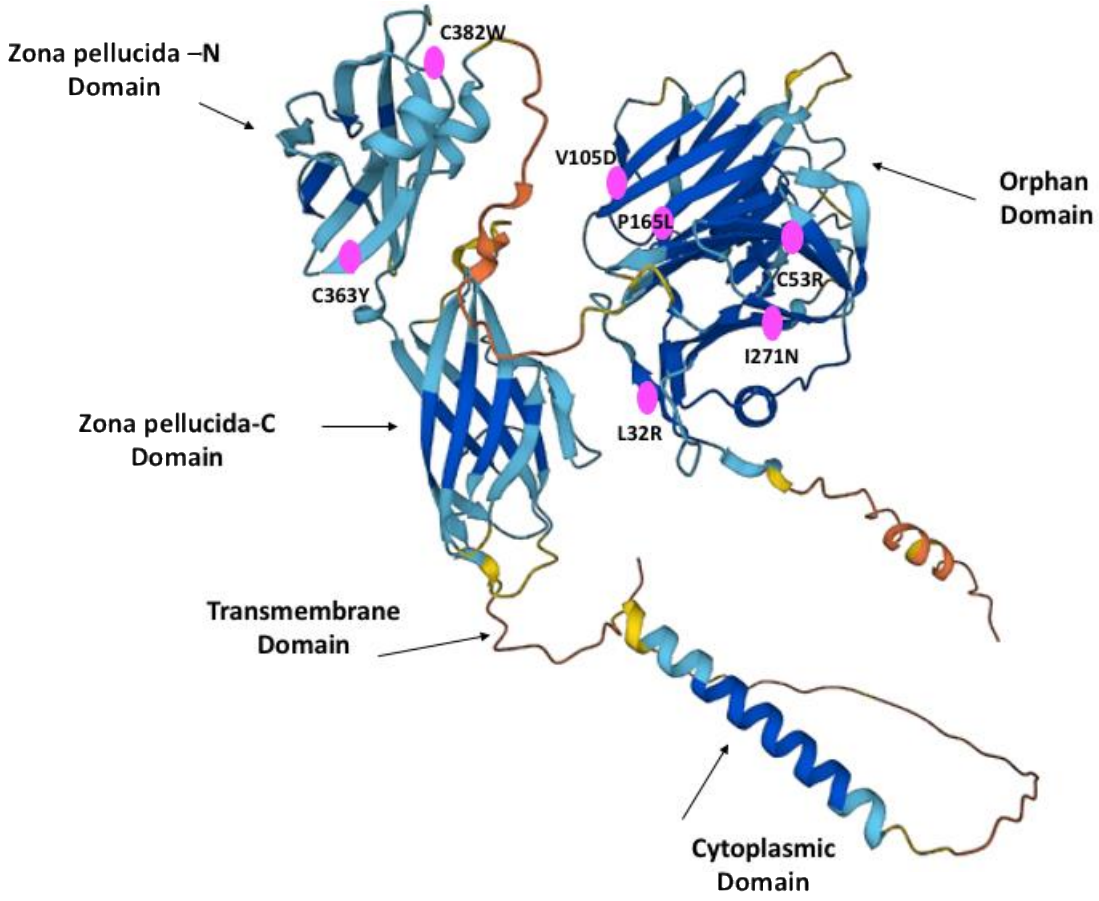

Figure 8: Predicted structure of endoglin WT and variants

The figure shows 3D ribbon structure of endoglin WT built by Alpha Fold protein structure database based on a homologous structure. Missense mutations examined in this study (P165L, V105D, L32R, C53R, I271N and C363Y are illustrated with a pink circle at their designated domain. 3D structure was generated by Alpha Fold protein structure database

1.4.7 Proposed Mechanisms for Some Pathogenic Endoglin Mutants

Newly synthesized proteins enter the ER through the translocon channel in the unfolded states, where they get modified, if required, and often simultaneously go through their folding pathways, a process that involves several molecular chaperones and enzymes.

Once they reached their proper conformation and assembly, properly folded and assembled proteins get dispatched to the downstream organelles in the cell along the secretory pathway to their final destinations (Braakman & Hebert, 2013). However, protein folding, posttranslational modifications and assembly are error prone and are affected by mutations, therefore it is tightly controlled by the ERQC mechanism which ensures that only properly folded proteins can reach their target destinations within the cell (Guerriero & Brodsky, 2012). The process can be complicated by disease-causing mutant variants of proteins that are structurally mis- or mal-fold and are, therefore, incapable of reaching their native conformation. Accumulation of these proteins disrupt ER homeostasis and the ERQC is triggered to resolve the problem through the activation of the unfolded protein response (UPR) (Hwang & Qi, 2018). The UPR is an adaptive process that activates a coordinated cellular and nuclear responses that reduce protein input, increase folding capacity and activate ERAD that degrades misfolded proteins through the proteasomal pathway (Sun & Brodsky, 2019). On the other hand, it became clear recently that some misfolded proteins aggregate in the secretory pathway are targeted and degraded by autophagy (Qi et al., 2017). Therefore, recently, the umbrella term ERALD (ER-to-lysosomes-associated degradation) for autophagic and non-autophagic pathways triggered by ERAD-resistant misfolded proteins, has been proposed (Fregno & Molinari, 2019).

The majority of endoglin mutations reported so far result in either unexpressed protein, dysfunctional protein localized at the plasma membrane or intracellular proteins trapped in the ER, which all together support the haploinsufficiency model for the general disease mechanism (Ali et al., 2011; Galaris et al., 2021; Marchuk, 1998). Nonetheless, the downstream mechanism after ER-retention remains to be elucidated for most of the ER-retained mutants. Therefore, in this project we set out to investigate the possible lysosomal and proteasomal degradation pathways for some ER-retained mutant variants of endoglin and compare them with the WT protein (Figure 9). Cellular and molecular interventions targeting degradation pathways for the purpose of rescuing mutant variants that are possibly functional could be a window for potential therapy.

Furthermore, some *in vitro* studies have shown that some missense mutants can form heterodimers with the wildtype giving rise to a possible dominant negative effect (Förg

et al., 2014; Mallet et al., 2015). I therefore in this thesis, examined the possible dominant negative effects of several missense mutants trapped in the ER as a major focus of my project.

1.4.8 Elucidation of Degradation Pathways in Biochemical Research

Investigating degradation pathways in biochemical research involves studying the turnover and fate of proteins or other biomolecules within a cell. One of the approaches that is widely used is the Inhibition or activation of degradation pathways. Specific degradation pathways can be inhibited or activated using pharmacological agents or genetic manipulations. For example, proteasomal inhibitors like MG132 or Epoximycin can be used to inhibit the proteasome and assess the role of this pathway in protein degradation (figure 9). MG132 is a peptide aldehyde-based proteasomal inhibitor that functions by irreversibly binding to the active sites of the proteasome, thereby blocking its proteolytic activity (Bo Kim et al., 2005; Xu et al., 2022). As a result, protein degradation by the proteasome is inhibited. By inhibiting the proteasome, MG132 allows the accumulation of specific target proteins, enabling researchers to investigate their degradation stability, and turnover mechanisms. Similarly, Epoximycin is a microbial secondary metabolite derived from the actinomycete strain Actinoplanes, and functions as a reversible and selective proteasomal inhibitor (Bo Kim et al., 2005). Epoximycin specifically binds to the β5 subunit of the proteasome, inhibiting its proteolytic activity (Figure9) (Bo Kim et al., 2005). This prevents the degradation of proteins targeted for proteasomal degradation via the chymotrypsin-like pathway (Myung et al., 2001) .

Bafilomycin, a widely used lysosomal inhibitor, blocks the function of the vacuolar-type H+-ATPase (V-ATPase), a proton pump responsible for acidifying the lysosomal lumen (Yuan et al., 2015) (Figure 9) (Yuan et al., 2015). By inhibiting the V-ATPase, bafilomycin disrupts the normal acidification process, resulting in an increase in the pH of the lysosomal environment. This elevated pH interferes with the optimal activity of lysosomal enzymes, which rely on an acidic pH for their functionality. Consequently, the impairment of lysosomal enzymes leads to a disruption in lysosomal degradation processes. As a result of the compromised lysosomal function, various substances including proteins, lipids, and cellular debris, accumulate within the cells.

Degradation pathways can also be investigated via inhibiting components of a specific degradation route in order to specify a particular degradation channel. In order to investigate the degradation pathway through which misfolded proteins are degraded, ERAD inhibitors such as Eeyarestatin (EerI) and Kifunensin (Kif) are often utilized (Figure9). EerI functions as an ERAD inhibitor by interfering with the retrotranslocation of misfolded proteins from the ER to the cytosol . This disruption of ERAD leads to the accumulation of misfolded proteins (Wang et al., 2010). However, EerI was shown to lack specificity and may interfere with other cellular compartments such as the Golgi apparatus and vesicular trafficking. This lack of specificity for ERAD can limit its utility in studying the specific role of ERAD in cellular processes. Treatment with Kif, a potent inhibitor of mannosidase I enzyme acts on the mannosidase I enzyme, which plays a role in the recognition process of misfolded glycoproteins (Wang et al., 2011). Inhibiting this early stage of ERAD substrate recognition is likely to inhibit the subsequent protein degradation pathway.

In order to evaluate the effects of protein degradation pathways, it is possible to conduct genetic manipulations that involve the suppression or elimination of specific genes associated with these pathways. For instance, the use of techniques like siRNA or CRISPR/Cas9 can be employed to induce knockdown or knockout of particular genes involved in ubiquitin ligases or autophagy-related processes. By doing so, it becomes feasible to uncover the functions of these genes in regulating protein turnover.

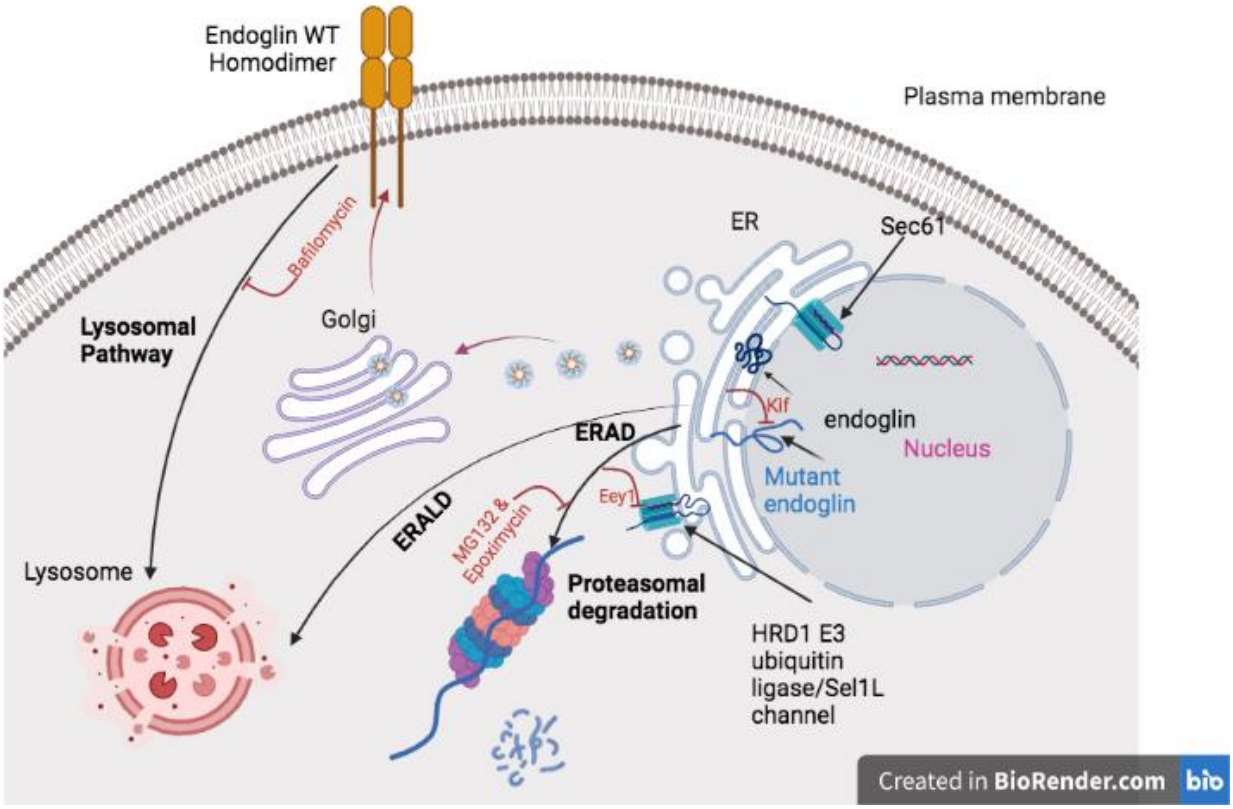

Figure 9: Endoglin proposed degradation pathways

This image is a schematic illustration of the biosynthesis and folding of endoglin WT (black) in the ER, trafficking to the Golgi apparatus for further modification and then its dispatching to the plasma membrane where it functions as a TGFβ co-receptor. It also illustrates our tested hypothesis in this thesis that mutant ER-retained endoglin (blue) gets degraded either through ERAD or lysosomally through ERALD. On the other hand, WT endoglin is likely to degrade through the lysosomal pathway. Proteasomal inhibitors (MG132 and Epoximycin), lysosomal inhibitors (Bafilomycin) and ERAD inhibitors (Eeyarestatin 1 (Eey1)) are indicated. Created using BioRender.com

1.4.9 HHT Molecular Mechanisms: Insights Beyond the Mutant Variants

The proposed mechanisms for HHT pathology including haploinsufficiency and the dominant negative effect has still not been able to account for the specific localization of vascular lesions presented in HHT patients. It is intriguing why the telangiectatic lesions appear on specific sites rather than all over the body. To resolve this paradox, a possible second hit that induces vascular malformation in specific sites has been proposed. Examples of a second hit effect are external triggers such as vascular injury, inflammation, acute infection, hypoxia, angiogenic stimuli, or a second somatic mutation in vascular lesions of HHT patients (Gallardo-Vara et al., 2019).

Over the years, the second-hit hypothesis has developed to account for the various factors, both environmental and genetic (such as modifier genes or somatic mutations), that may contribute to the development of complex diseases characterized by phenotypic heterogeneity.

In the case of HHT, it has been recognized that as patients age, disease progression gets worse (Gonzalez et al., 2019). It has also been observed that older patients present with higher number of telangiectases in the hands and lips. This observation is in agreement with the fact that older patients experienced accumulated cutaneous insults over the years, compared to younger patients (Geisthoff et al., 2020). Interestingly, the study found that HHT patients developed more telangiectases on their dominant hand, which suggest that mechanical stress induced by manual work may contribute to the increased number of telangiectases (Geisthoff et al., 2020).

Vascular endothelial growth factors known to promote angiogenesis and vascular modelling were also shown to be elevated in telangiectases and plasma of HHT patients (Sadick et al., 2005). Experimental animal models have also demonstrated that a mouse model with conditional mutation in *ENG* only developed AVMs when an angiogenic stimuli is combined with heterozygous mutation (Gallardo-Vara et al., 2019).

Inflammation has also been considered as an external stimuli that contributes to the formation of telangiectatic lesions in HHT patients. An interesting observation is that both HHT skin telangiectases and internal AVMs display a proinflammatory cell infiltrate that includes lymphocytes and macrophages (Zhang et al., 2016). This suggests that the dysregulation of the vascular repair process, which involves both endoglin function and leukocyte infiltration, could play a role in the formation of AVMs in HHT1. In fact, it has been reported that endoglin is upregulated in endothelial cells following inflammation or injury. These findings implicate endoglin in the recruitment of leukocytes to mediate vascular repair in HHT patients (van Laake et al., 2006). Moreover, the presence of a genetic second-hit, such as a somatic mutation in the normal HHT allele, when combined with an environmental trigger or the presence of modifier variants, can significantly accelerate the disease progression. In all cases, the outcome is

distorted endothelial cell function, which results in the manifestation of the disease symptoms, such as formation of telangiectases and AVMs.

It has been also postulated that second hits may be tissue specific and that is due to the organ-specific location of phenotype in HHT patients. In agreement with this notion, a positive correlation has been found between the basal level of endoglin expression in specific organs and the manifestation of the disease phenotype. For example, endoglin expression on the skin, which is the first site of HHT presentation, was found to be significantly low compared to other organs (Galaris et al., 2021). Hence it was proposed that organs exhibit abnormal functions only if endoglin levels fall below a critical threshold.

In conclusion, there are still major gaps in our understanding, particularly in identifying other factors that contribute to the development of clinically significant symptoms in HHT and how this knowledge can be applied in clinical practice. Further research is necessary to better comprehend the pathological mechanisms of HHT and to translate this knowledge into preventive clinical measures and treatments.

1.4.10 The ER Protein Folding Quality Control and ERAD: Components and Mechanisms

The ER has adopted a highly sophisticated, stringent and conserved quality control mechanism known as ER- associated protein degradation (ERAD) to dispose of a diverse range of proteins including improperly folded secretory and membrane proteins, orphaned subunits of protein complexes and some endogenous proteins (Preston & Brodsky, 2017; Sun & Brodsky, 2019). ERAD is a complex process that involves the coordination of the functions of many proteins in both the ER, and the cytoplasm with input from the nucleus through UPR, a cellular adaptive mechanism to resolve ER stress (Christianson & Ye, 2014). In order for a misfolded protein to be discarded, it needs to be recognized, retrotranslocated into the cytosol, and then extracted through the ER membrane to be degraded in the cytosol by the ubiquitin/proteasomal system (Figure 10) (Wu & Rapoport, 2018).

Newly synthesized proteins that successfully attain native conformations and assemble into complexes, if needed, with the assistance of ER resident molecular chaperones are usually allowed to be incorporated into COPII vesicles and transported further to reach their final destinations and organelles within the secretory pathway or be secreted outside the cell (Needham & Brodsky, 2013). However, fully or partially misfolded proteins and orphaned proteins are retained in the ER and then usually get degraded by the ubiquitin proteasomal systems (Sun & Brodsky, 2019). On the other hand, it became clear recently that some misfolded proteins aggregate in the secretory pathway and are targeted for degradation by autophagy (Qi et al., 2017). Therefore, recently, the umbrella term ERALD (ER-to-lysosomes-associated degradation) for autophagic and non-autophagic pathways triggered by ERAD-resistant misfolded proteins, has been proposed (Fregno & Molinari, 2019). Figure 10 shows the possible fates and stages of ER misfolded proteins. Proteins that are structurally mutated are likely to fail reaching the proper conformation and will possibly be transiently trapped in the ER. The length of stay in the ER before the decision to degrade the misfolded or malfolded protein varies from one protein to another. Accumulation of misfolded proteins in the ER is often cytotoxic and may cause ER stress leading to an array of metabolic, immune and degenerative diseases (Hetz, 2012; Sha et al., 2011; Zito, 2019). In addition, unresolved mutated proteins may form aggregates that disrupt ER homeostasis and trigger UPR that activates expression of molecular chaperones that can process aberrant proteins as well as transcription factors that attenuate protein translation to reduce protein synthesis load on the ER (Nishikawa et al., 2005).The first and perhaps the most crucial step in ERAD is the recognition of an ERAD substrate in the highly crowded ER environment that harbors a whole spectrum of newly synthesized proteins in varying stages of their folding, oligomerization and post-translation modifications. Therefore, commitment to degrade a particular protein through ERAD is highly specific and must, therefore, be very tightly controlled. In order to differentiate between permanently misfolded and partially folded native proteins, both protein species are kept in their soluble form bound to the mammalian ER molecular chaperone Hsp70 (BiP in mammals). BiP recognizes the exposed hydrophobic regions of improperly folded protein species and plays a key role in folding, or otherwise, disposal through the ERAD pathway due to the prolonged

association with BiP (Stevenson et al., 2016). On the other hand, N-linked glycoproteins have a characteristic and well-defined glycan moiety of three glucose, nine mannose, and two N-acetylglucosamine residues (Glc3–Man9–GlcNAc2). Glucosidases I and II (GLU I and GLUII) catalyze the cleavage of two glucose residue from a triglycosylated form of protein-bound oligosaccharide and hence facilitate the binding of ER molecular chaperones to monoglycosylated glycoproteins ($GlMan_9GlcNac_2$) which in turn play a crucial role in glycoprotein folding and processing (Ruggiano et al., 2014; Tax et al., 2019). Glycoprotein lectins such as Calnexin and Calreticulin (CNX/CRT) facilitate protein folding through repeated cycles of interaction with nascent proteins via their high binding affinity to the mono-glucose residue. However, if the protein fails to fold properly these two chaperones recognize specific N-linked carbohydrate moiety structures that are associated with glycoproteins and target them for ERAD. Terminally misfolded glycoproteins are extracted from CNX/CRT by members of the EDEM (ER degradation-enhancing α-mannosidase-like protein) family such as (EDEM1-3) and ER mannosidase I. Mannosidase I removes one mannose residue from the glycoprotein in a process known as “mannose trimming” that facilitates substrate transfer from calnexin to EDEM, a step that signals initiation of the ERAD mechanism (Nishikawa et al., 2005; Oda et al., 2003). OS-9 and XTP3-B are other ER resident lectins that bind luminal misfolded glycol proteins to the Hrd1 E3 ligase through the transmembrane adaptor protein (SEL-1L) (Christianson et al., 2008). Once a mutant protein is recognized and selected as a substrate for disposal by ERAD, it is transferred out of the ER to the cytoplasm for degradation through a process referred to as retrotranslocation or dislocation. Despite the fact that the mechanisms by which a mutant protein is channeled through the ER membrane to the cytosol is still not clear, it has been established that E3 ubiquitin ligases coordinate the execution of this crucial step (Ruggiano et al., 2014). The best-characterized E3 ligases in mammalian cells are HRD1 and Glycoprotein 78(GP78), which are both ER multi spanning membrane proteins with a RING domain responsible for the ligase activity in the cytoplasm (Bernasconi et al., 2010; Joshi et al., 2017). In yeast, it has been characterized that E3 ligase complexes are specific to the location of the lesion (mutation) on the ERAD substrate. For example proteins with cytoplasmic lesions (ERAD-C) are degraded through the Doa10 E3 ligase complex,

whereas proteins with structural misfolding in their luminal (ERAD-L) or intramembrane (ERAD-M) are degraded through the Hrd1 E3 ligase complex (Vashist & Ng, 2004). Asi E3 ligase complex has recently been identified and its substrates included soluble and intramembrane proteins (Foresti et al., 2014; Stevenson et al., 2016). In mammalians, correlation between the type of ERAD substrate and choice for degradation pathway has also been identified in few cases, however it is still not clear what dictates a particular pathway for mammalian ERAD substrates and that is primarily due to the complexity of the retrotranslocation channels and its associate proteins (Lemus & Goder, 2014). Unlike in yeast, several mammalian E3 ligases, beside Hrd1 and GRP78, have been implicated in the ERAD substrate dislocation such as RMA1, TEB4, RFP2,TRC8, Kf-1, RNF170 and Nixin/ZNRF4 (Claessen et al., 2012). Other ER trans-membranous proteins such as Derlin-1, Derlin-2 and Derlin-3 (Der1p family) also associate with the E3 ligases and other ERAD factors to form a complex retrotranslocation channel spanning the ER membrane. In mammals, Derlins play a major role in the retrotranslocation of luminal substrates, however their exact function remains speculative (Christianson & Ye, 2014; Huang et al., 2013).

It has been identified that ERAD substrate dislocation is driven by a cascade of ubiquitination enzymatic activities that labels defective proteins that should undergo proteasomal degradation with a chain of four or more ubiquitins attached to lysine 48 to be recognized by the 19S cap of 26S proteasome (Preston & Brodsky, 2017). Ubiquitin ligase enzyme (E1) activates a ubiquitin-conjugating enzyme (E2), of which there are 40 enzymes. They together work in conjunction with a ubiquitin ligase (E3), of which there are 650 enzymes, to transfer ubiquitin to the selected ERAD substrate (Caldeira et al., 2014; Christianson & Ye, 2014). The attachment of a poly-ubiquitin chain to ERAD substrates triggers the recruitment of AAA+ ATPase Cdc48 (p97/VCP in mammals) that provides the energy for the extraction of nearly all EAD substrates (Stolz et al., 2011). However, the fact that p97/VCP is a cytoplasmic protein means it can only interact with luminal ERAD substrates after they are partially out of the ERAD translocon channel, which raises many questions about the origin of the energy that drives the initiation of the retrotranslocation process(Olzmann et al., 2013). Mammalian E2s such as UBE2J1, UBE2J2 and UBE2G2 have also been implicated in the dislocation process, however

their specificities towards a particular E3 ligase have not yet been established (Claessen et al., 2012). Ubiquitination in ERAD is also controlled by the opposing effect of Deubiquitinases (DUB) that remove ubiquitin chains conjugated with ERAD substrates. DUBs were found to be in close association with p97/VCP and 26S proteasome, which suggests their role in substrate dislocation and proteasomal function. It has been proposed that this association facilitates the threading of unfolded proteins to be degraded in the proteolytic chamber of 26S proteasome (Ernst et al., 2011; Pickart & Cohen, 2004).

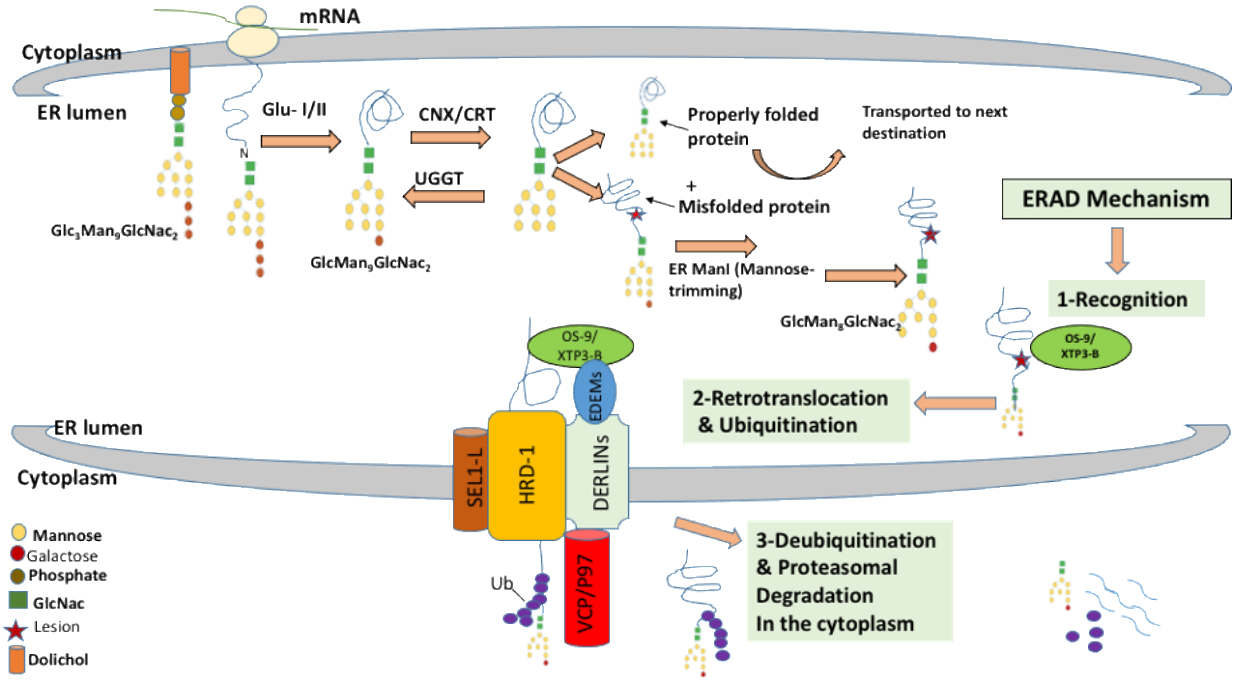

Figure 10: ERAD mechanism for misfolded glycoprotein through the HRD-1 E3 ligase/SEL-1L complex

Triglycosylated form of protein-bound oligosaccharide ($Gl_3Man_9GlcNac_2$) is processed by glucosidase enzymes (GluI/II) that cleaves off two glucose molecules. This is followed by cycles of interaction between the nascent protein and lectins such as Calnexin and Calreticulin (CNX/CRT), that binds specifically to monoglucosylated oligosaccharides ($GlMan_9GlcNac_2$) and ensure the proper folding of newly synthesized protein. This cycling effect is generated by the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT), that transfers back a glucose residue to the improperly folded protein to enable further encounters with the ER chaperones (CNX/CRT). A properly folded protein is then released after the cleavage of the remaining glucose molecule. Properly formed protein is dispatched to its functional destination, while misfolded protein that cannot reach its mature form will undergo mannose cleavage by ER α1,2-mannosidase I (ERMan1), which produces Man8GlcNAc2. Terminal mannose cleavage (α mannose) function as a recognition signal for ERAD lectins OS-9 and XTP3-B that recognize and binds to exposed mannose residues after cleavage of α mannose. The three chaperones EDEM1, OS9 and XTP3-B function together as recognition complex that interacts with misfolded proteins and the HRD-1/SEL-1L retrotranslocation channel. Derlins which are candidates for the translocon channel also interacts with the EDEMs and facilitates the interaction of EDEMs with cytosolic AAA-ATPase p97, that provides ATP hydrolysis for successful extraction of mutant proteins. Retrotranslocation is coupled by Ubiquitination, a process that targets proteins for degradation by 26S proteasome by tagging them with ubiquitin chains (Gariballa & Ali, 2020).

1.4.11 ERAD as a Major Human Disease Mechanism

Mutations altering the amino acid sequence or structure of a protein can induce protein misfolding. Such mutations can impede the protein's ability to fold appropriately or promote protein aggregation, resulting in functional deficits or potential harm. ERAD

identifies and eliminates misfolded proteins that arise from these mutations, thereby ensuring their degradation (Fregno & Molinari, 2019).

The implication of the ERAD mechanism has been reported in several monogenic diseases such as cystic fibrosis (CF), which is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. Over 2000 mutations in this gene have been identified to cause CF, a disease that is mainly characterized by impaired lung function and gastrointestinal tract alterations (Veit et al., 2016). Mutations in the CFTR gene encompasses frameshift, nonsense and splice site mutations that introduce a premature termination codon, resulting in reduced protein expression. Insertion/deletion mutations as in (Phe508del) have also led to a misfolded protein being retained in the ER and prematurely degraded through the proteasomal pathway (Balch et al., 2011). ERAD has also implicated in Robinow Recessive Syndrome (RRS), a rare genetic disease affecting 1 in 500,000 individuals, and characterized by skeletal abnormalities (Chen et al., 2005). Point mutations in the causative gene; *ROR2*, encoding an orphan receptor tyrosine kinase, results in a misfolded protein that is targeted by the ERQC mechanism for premature proteasomal degradation through ERAD (Chen et al., 2005).

Truncated proteins resulting from nonsense mutations often exhibit impaired or lost functionality and can pose potential harm to the cell. The ERAD mechanism specifically recognizes and directs truncated proteins towards degradation in order to prevents their accumulation and potential toxic effects on the cell. For example, ERAD-mediated degradation of truncated TDP-43 protein, which is implicated in ALS (Amyotrophic Lateral Sclerosis), reduces the formation of TDP-43 aggregates and thereby diminishes their associated toxicity (Medinas et al., 2017; Nishitoh et al., 2008) (Nishitoh et al., 2008; Nishitoh et al., 2002)

Mutations in the amyloid precursor protein (APP) gene, associated with familial forms of Alzheimer's disease, have been identified(Kaneko et al., 2017). These mutations can result in abnormal processing of amyloid-beta (Aβ) peptides, which have a tendency to aggregate and form plaques in the brains of individuals with Alzheimer's disease. ERAD mechanism plays a crucial role in recognizing and directing misfolded or aggregated forms of APP and Aβ peptides for degradation. Consequently, ERAD helps maintain the

balance of these proteins, reducing the accumulation of amyloid plaques and their associated burden in the brain (Kaneko et al., 2017).

Lysosomal storage disorders such as Gaucher, Tay-Sachs and infantile GM1-gangliosidosis diseases caused by missense mutations that affects the native folding of lysosomal enzymes are also candidates for proteasomal degradation via the ERAD mechanism (Mohamed et al., 2020; Wang et al., 2011). Our group has also demonstrated the implication of ERAD in the pathology of Dysequilibrium syndromes and hypercholesterolemia associated with missense mutant variants of Very Low-Density Lipid Receptor (VLDLR) and LDLR, respectively (Kizhakkedath et al., 2018; Kizhakkedath et al., 2019).

This wide-spread involvement in single gene disorders and common conditions is simply because the ER is a central organelle and the entry point to the secretory pathway with almost a third of all the cellular proteins encoded in the human genome are targeted to the ER in transit to their final cellular destinations and are therefore subjected to the highly stringent quality control system within the ER (Sun & Brodsky, 2019). On one hand, failure of ERAD mechanism to degrade accumulated mutant proteins induces ER stress which often leads to cellular toxicity and possibly cell death. On the other hand, degradation of a mutant, but possibly functional, protein is also likely to deprive the cell of an important functional protein leading to loss of function phenotypes which have been observed for numerous hereditary diseases, as mentioned above. HRD1 E3 ubiquitin ligase has been shown to prevent the accumulation of Amyloid Precursor Protein (APP), implicated in the pathology of AD, (Kaneko, 2016). Reduced levels of HRD1 has been observed in cerebral cortex tissues of patients with AD, which suggests an implication of ERAD in the pathogenesis of this disease (Kaneko et al., 2002). Parkin2 (*PARK2*), is another ERAD ubiquitin ligase that is involved in the pathogenesis of familial Parkinson Diseases (PD) (Ishikawa & Tsuji, 1996). Mutations in *PARK2*, including missense, nonsense, frameshift and splice site mutations, leads to accumulation of its substrates in the ER leading to ER stress- induced neuronal cells death (Imai et al., 2001). We and others have demonstrated in numerous studies the involvement of the ERAD mechanism in the pathogenesis of monogenic diseases such as autosomal dominant HHT1, HHT2 and PAH, that are caused by mutations in *ENG*, *ACVRL1* and

BMPR2, respectively (Ali et al., 2011; Hume et al., 2013; John et al., 2015). The mutant proteins encoded by the three genes are transmembrane receptors in the TGFβ signaling superfamily. The focus of this project is the elucidation of the molecular and cellular mechanisms involved in the pathogenesis of HHT1 caused by missense mutations in endoglin that are retained in the ER.

Chapter 2: Methods

2.1 In Silico Analysis of Endoglin Variants P165L and V105D

We have used AlphaFold protein structure database (https://alphafold.ebi.ac.uk/) for the presentations of 3D ribbon structure of WT endoglin and all mutant variants studied in this thesis. AlphaFold database prediction tools have been validated in the14th Critical Assessment of protein Structure Prediction (CASP14), in which they were highly rated by the reviewers (Jumper et al., 2021; Varadi et al., 2022)

We have also utilized HOPE online software (https://www3.cmbi.umcn.nl/hope/), which is a fully automated protein modelling program, for the prediction of functional and structural effects for all endoglin mutant variants studied here (Venselaar et al., 2010).

2.2 Cell Culture, Transient Transfection and Treatments

Human Embryonic Kidney 293 cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen), penicillin (10 U/ml) and streptomycin (100 µg/ml) at 37°C with 5% CO_2. For transfection experiments, cells were grown in 6-well tissue culture plates and transfected with 1µg plasmid DNA using FuGENE HD transfection reagent. Co-transfection with 0.5 µg plasmid vector harboring Red Fluorescence Protein (RFP) (Thermo Fisher Scientific) was carried out as a transfection efficiency control for transient transfection of plasmid constructs carrying WT endoglin and mutant variants.

For cycloheximide chase assays, stably transfected HEK293 cells were treated with cycloheximide (100 µg/ml) and harvested at specific time points (0, 2, 4, 8, 16 and 24 hours). Cells were then lysed in RIPA buffer and kept at -80°C for Western blot experiments.

2.3 Degradation Pathways Inhibitors Treatments

For treatments with proteasomal, lysosomal or ERAD inhibitors, Stably transfected HEK293 cells were grown in DMEM with a supplement of 10% FBS, penicillin (10 U/ml) and streptomycin (100 µg/ml) until they reach 60-70% confluency. Cells were then serum starved for 4-8 hours followed by incubation in serum free medium

containing the indicated proteasomal inhibitors (MG132 at 10μM and Epoximycin at 100nM), ERAD inhibitors (Eeyarestatin I at 5 μM/ and Kifunensine at 50 nM), and lysosomal inhibitors (Bafilomycin at 200 nM). Optimal concentration for these inhibitors in HEK293 cell lines were optimized in a previous study carried out by our research group (Kizhakkedath et al., 2019).

2.4 Protein Extraction, Western Blotting Analysis and Immunoprecipitation (IP)

After cells were harvested and pelleted, protein extraction was carried out using RIPA lysis reagent (Sigma) supplemented with protease inhibitors cocktail (Sigma fast protease inhibitor cocktail). Cell lysates were then quantified using Bicinchoninic Acid protein Assay (BCA kit, Pierce), according to manufacturer's protocol. Equal amounts of protein were mixed with Laemmli loading buffer, heated to 95°C for 5 minutes and then resolved on an SDS-PAGE at concentrations relevant to the protein sizes. However, endoglin was found to aggregate at the top of the acrylamide gel at 95°C, therefore cell lysates were mixed with Laemmli buffer plus Dithiothreitol (DTT, 5 mM) and heated at 55°C for 10 minutes prior to loading into the gel. This was followed by blotting onto a PVDF page and then probed with the respective antibodies at an optimized dilution. Detection was performed using Enhanced Chemiluminescence Plus reagent (ECL plus, Pierce) and then it was visualized using Typhoon FLA 9500 Imager (GE Healthcare Biosciences). ImageJ software was used for densitometric quantification analysis of immunoblots generated.

For immunoprecipitation, stably transfected HEK293 cells were lysed in IP lysis buffer (Pierce Inc.) supplemented with protease inhibitors cocktail (Sigma fast protease inhibitor cocktail). After total protein extraction and quantification (as described above), equal amounts of cell lysates were incubated with anti-HA agarose beads (Pierce) for 2 hours at 4°C. Beads containing the immunoprecipitated proteins were collected by centrifugation and washed three times with lysis buffer. Proteins were eluted from the beads by boiling in Laemmli sample buffer to be used for western blotting.

2.5 Triton X-100 Solubility Assay

The assay was carried out as in (Houck et al., 2014). In short, harvested cells were lysed in TBS-Triton (50mM Tris-Cl, 150mM NaCl, 1% Triton X-100, pH 7.6) and separated into soluble fraction (supernatant) and aggregated fraction (pellet) via 20,000 g centrifugation for 15 minutes at 4ºC. Thirty µg of cell lysates were added to SDS sample buffer with 1.25% β-mercaptoethanol and then resolved on an SDS-PAGE. GAPDH was used as a marker for the soluble fraction and Histone H3 was used as a marker for pelleted fraction.

2.6 Generation of HEK293-HRD1 Knockout Cell Line using CRISPR-Cas9 Gene Editing

KN2.0 non-homology mediated CRISPR kit (Origene inc.) was used for *HRD1* gene Knockout. The gene specific gRNA carries the sequence (ACTGTGGTGTACCTGACCAA) leads Cas9 enzyme to cut the target genome, and the cutting site is repaired by the integration of predesigned linear donor containing a puromycin resistant gene for selection of cells that have the linear donor encoding the reporter gene; Green Fluorescence Protein (*GFP*) integrated (Figure 11). The gRNA vector plus the DNA donor were transfected into the cells using FuGENE HD transfection reagent. Scrambled gRNA was used as negative control. 1µg of the gRNA vectors (or scramble control) was diluted in 50 µl of Opti-MEM (Thermofisher Scientific), then 1 µg of the donor DNA is added and vortexed thoroughly before adding 6µl of the FuGENE HD transfection reagent. The mixture is then added -drop-wise- to the cells plated on the culture plate. Cells were then incubated in a 5% CO_2 incubator for 48 hours before splitting in (1:10) ratio. Cells were passaged 5 to 6 times before treatment with the pre-determined kill dose for puromycin (0.7 µg/ml) for seven days for optimal selection of positive clones. Multiple splitting before puromycin treatment is essential for the elimination of donor DNA that is not incorporated into the DNA, which could generate false positive results. Single cells were then seeded in a 96 well plated using serial dilution of cell culture. After two weeks of observation single cell wells were marked and grown on 6 well plates for DNA/RNA and protein extraction. Bi-allelic gene perturbation on the DNA level has been confirmed using Sanger sequencing and gene knockout on the protein level was validated using immunoblotting against HRD1.

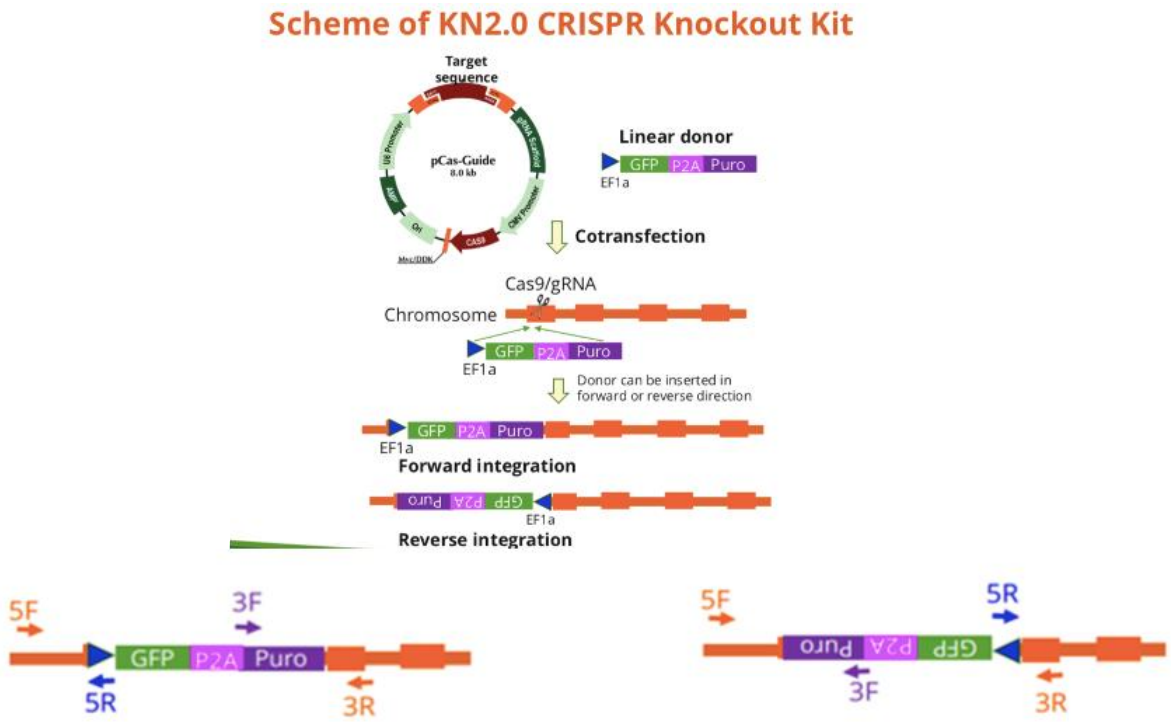

Figure 11: Scheme of CRISPR Cas9 knockout system

(https://www.origene.com/products/gene-expression/crispr-cas9/knockout-kits)

2.7 Immunocytochemistry

HEK293 cells, stably transfected with pcDNA3.0 expression vector harboring the HA-tagged WT endoglin or the missense mutant variants P165L and V105D or transiently transfected cells, were grown on coverslips. Cells were then fixed by methanol at –20°C for 5 minutes. Fixed cells were washed three times with PBS and incubated in blocking solution (1% BSA in PBS) for 60 minutes at room temperature. For immunofluorescence staining, cells were incubated with the primary antibody for 1 hour at room temperature, then washed three times with PBS. After washing, cells were incubated with fluorescent-labelled secondary antibody, followed by washing three times for five minutes with PBS. Cover slips were then mounted with immunofluor medium on the microscope slide, ready for detection and imaging on the confocal fluorescence microscope.

2.8 Antibodies

Antibodies for Western blot analysis: rabbit monoclonal anti-HA-tag (Cell Signaling Technology, at 1:1000 dilution), rabbit monoclonal anti-SYVN1 (Cell Signaling Technology, at 1:1000 dilution), mouse monoclonal anti-GAPDH (Abcam, at 1:2500

dilution) rabbit monoclonal Anti-HA (Cell Signaling, at 1:1000 dilution), mouse monoclonal anti-RFP (Thermo Fisher Scientific, at 1:1000 dilution), rabbit anti-endoglin P3D1 (Santa Cruz Biotechnology, at 1:200 dilution), anti-Mouse IgG Peroxidase antibody (Sigma Aldrich, at 1:40,000 dilution), anti-Rabbit IgG Peroxidase antibody (Sigma Aldrich, at 1:30,000 dilution) and mouse monoclonal anti-Myc (Cell Signaling Technology, at 1:500 dilution).

Antibodies for immunofluorescence: mouse monoclonal anti-HA-tag (Cell signaling Technology, at 1: 200 dilution), rabbit polyclonal anti-calnexin (Santa Cruz Biotechnology, at 1 : 200 dilution), rabbit anti-Histone-H3 (Cell Signaling Technology, at 1:1000 dilution), mouse monoclonal anti-HA (Cell Signaling Technology, at 1: 200 dilution), Alexa Fluor 568-goat anti-mouse IgG (Molecular Probes, at1:1000 dilution) and Alexa Fluor 488-goat anti rabbit IgG (Molecular Probes, at 1:000 dilution) and goat polyclonal anti-calnexin (Santa Cruz Biotechnology, at 1:50 dilution)

2.9 Fluorescence-Activated Cell Sorting (FACS)

HEK293 cells stably transfected with WT endoglin and mutant variant P165L and V105D were grown until 70% confluent and then washed, dissociated and pelleted down using 1500 RPM centrifugation for 5 minutes, at 4℃. Cells were then blocked in Blocking solution (0.5% BSA in PBS), centrifuged and incubated with anti-endoglin P3D1antibody on ice for 45 minutes. Cells were then washed and centrifuged to pellet three times. This is followed by incubation with Anti-mouse 488 secondary antibody at a dilution of 1:1000 for half an hour, then washed three times and pelleted down. Cells were then suspended in 1 ml of PBS and transferred to FACS tubes with filter cap for FACS analysis. Untreated HEK293 cells were used for gating. Cells transfected with an empty pcDNA3 vector were used for negative control.

2.10 Statistical Analysis

Statistical analysis between each group and control was conducted using non-parametric Mann-Whitney test, as the sample size was small and some of the data was not normally distributed. For the sets of data that was normally distributed according to normality tests, t-test was used for statistical analysis. GraphPad Prism software was used for both

tests. Chi-square and Fisher's Exact tests were used for determining the significance between categorical variables (SPSS software). In all graphs, error bars represent Standard Error of the Mean (SEM) from biological replicates indicated as the number (n) on the figure legends. Significance was denoted by the P value as follows (*) $P \leq 0.05$; (**) $P \leq 0.01$; (***) $P \leq 0.001$.

Chapter 3: Results

3.1 Protein Modelling Reveals Possible Structural and Functional Defects in ER-Retained V105D and P165L Endoglin Variants

In an attempt to better understand the basis of misfolding and ER retention of the two ER-retained endoglin variants V105D and P165L, we used HOPE protein modelling which could shed light on the possible structural effects of the missense mutations in endoglin and the possible consequences on the protein's biological function (Figure 12A). Endoglin variant V105D carries a point mutation that result in a substitution of amino acid Valine (V) at position 105 (outlined with red borders) to Aspartic Acid (D), (Figure 12B). The new amino acid Aspartic Acid (D) is bigger and carries a negative charge compared to the wild amino acid which is smaller and has a neutral charge. WT residue (V) is also reported to be very conserved in that position within a stretch of residues annotated in Uniport as required for interaction with BMP9 ligand. HOPE has also predicted that substitution of Proline (P) at position 165 to Leucine (L) in endoglin-P165L variant to be damaging (Figure 12C). Both variants fall in the Orphan domain where two conserved disulfide bridges involving C30–C207 and C53–C182 are located (Saito et al., 2017). The structural disorder caused by the P165L variants is predicted to disturb the cysteine bridge in this domain. The possible loss of cysteine bonding probably accounts for both distortion of the 3D structure of the protein as well as exposure of buried hydrophobic residues and possible formation of new cystine bonding that may cause mutant protein misfolding and aggregation.

An additional bioinformatic data we have utilized in order to assess the impact of the amino acid substitution is the Grantham distance, which is a quantitative metric developed by Grantham in 1974 to assess the differences in properties, such as polarity, composition, and volume, between different amino acids (Grantham, 1974) . The larger the Grantham distance, the greater the dissimilarity between the two amino acids in terms of these properties. Grantham distances of 50-60 are generally considered "conservative", 60-100 "non conservative" and great than 100 is considered "radical" (Tavtigian et al., 2008). Higher Grantham distances indicates greater dissimilarity between amino acids, suggesting a potentially more significant impact on protein

structure and function when substitutions take place. In the case of endoglin variants P165L and V105D Grantham distance between P and L is (98) and between V and D (152), which are both suggestive of a significant impact on the protein structure and function.

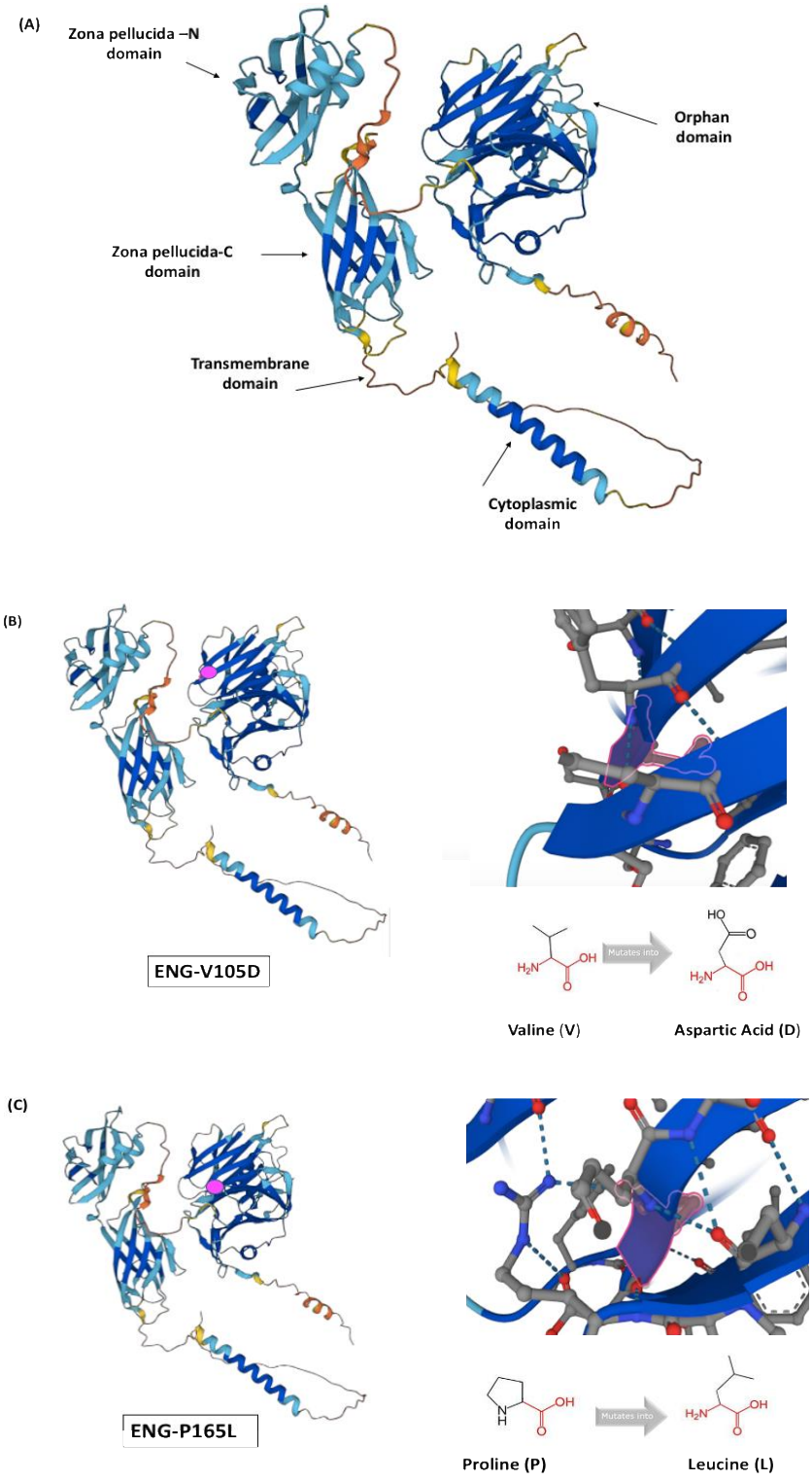

Figure 12: Predicted structure of endoglin WT and variants V105D and P165L

(A) shows 3D ribbon structure of endoglin WT built by Alpha Fold protein structure database. Predictions are based on homologous protein structures. (B) It shows mutation of amino acid variants Valine (V) to Aspartic acid (D) depicted with pink circle at the site of mutation. Focus on mutation site: The mutated residue is highlighted with red borders (C) Mutation of amino acid variants Proline (P) to Leucine (L) denoted with a pink circle. Focus on mutation site: The mutated residue is highlighted with red borders.

3.2 Endoglin Mutant Variants P165L and V105D Exhibit ER Retention in Endothelial Cells, Similar to HEK293 Cell Line

We have demonstrated in a previous study that missense mutant variants L32R, V49F,C53R, V105D, A160D, P165L, I271N, C363, A308D and C382W, transiently transfected into HeLa and HEK293 cells, are trapped in the ER and failed to traffic to their usual cellular functional location at the plasma membrane (Ali et al., 2011). These results had been demonstrated using Western blotting and immunofluorescence. techniques

In this study, stably transfected HEK293 cells expressing WT endoglin and the two ER-retained mutant variants P165L and V105D were generated as described in (Gariballa et al., 2022). In order to validate the relevance of our results using HEK293 cell line as a cellular model to study endoglin subcellular localization, trafficking and degradation, we investigated their subcellular localizations in endothelial cells, where endoglin is predominantly expressed. I have utilized immortalized human aortic endothelial cells (Telo-HAEC) for this purpose, as aortic endothelial cells are reported to express endoglin (Galaris et al., 2021). Telo-HAEC cells were transiently transfected with pcDNA3.0 expression vector harboring HA-tagged WT endoglin and mutant variant P165L and V105D as described in the methods section. Cells were then processed for immunofluorescence staining and imaging using fluorescence confocal microscopy. Cells were immunostained against endoglin- HA tag (Red) (Figure 13 panels (i) and (iv) and the ER marker; Calnexin (Green) (Figure 13 Panel ii). As expected, WT endoglin localized to the plasma membrane. In contrast, mutant variants P165L and V105D colocalized predominantly with the ER marker (calnexin) (Figure 13 panels iii). To further confirm the plasma membrane localization of the WT, Telo-HAEC cells were co-transfected with a vector harboring GFP-hRAS gene expressed as a plasma membrane marker (green) (Panel (v)). WT endoglin predominantly colocalized with the GFP-HRAS (Figure 13 Panel (vi)), while the two mutants did not show pattern of colocalization with the GFP-hRAS plasma membrane marker (Figure 13 panels (vi).

These results confirm that the subcellular localization and trafficking of endoglin WT and mutant variants P165L and V105D in Telo-HAEC endothelial cells is similar to that observed in HEK293 cells. One of the major advantages of using HEK293 cells as a

cellular model for this disease is its deficiency in endoglin which facilitates proper investigation of the trafficking of transfected variants without an interference from the indigenous protein expressed by the cells. Moreover, HEK293 cells have high transfection efficiency unlike Telo-HAEC cells which showed very low transfectability with various transfection agents including FuGENE and GenexPlus. Similarly, unsatisfactory transfection levels were obtained using electroporation transfection method. In addition, protein folding, and quality control is a highly conserved process and present in all eukaryotic cells. Therefore, HEK293 cells seemed to be suitable model especially for protein analysis experiments.

Table 4 Summarizes transfection efficiencies using various transfection methods optimized for transfecting HA- tagged pcDNA3.0 vector harboring WT endoglin and mutant variant P165L and V195D into HEK293 or TELO-HAEC cell lines. Factors that can affect the transfection efficiency including cell type, size of the construct, amount of DNA transfected, and transfection agent used, have all been taken into consideration for the optimization of transfection protocol. Nonetheless, TELO-HAEC cells recorded very low transfection efficiency that only few transfected cells were observed under the microscope .

Table 4: Transfection efficiencies recorded for HA-pcDNA3.0 vector harboring WT endoglin or variants

Cell Type	**Transfection Method**	**Transfection Efficiency-Approximate (%)**	**Toxicity**
HEK 293	FuGENE	80	Low
HEK 293	Genex Plus	70	Moderate
TELO-HAEC	FuGENE	0	Low
TELO-HAEC	GenexPlus	3	Moderate
TELO-HAEC	Electroporation	2	Moderate

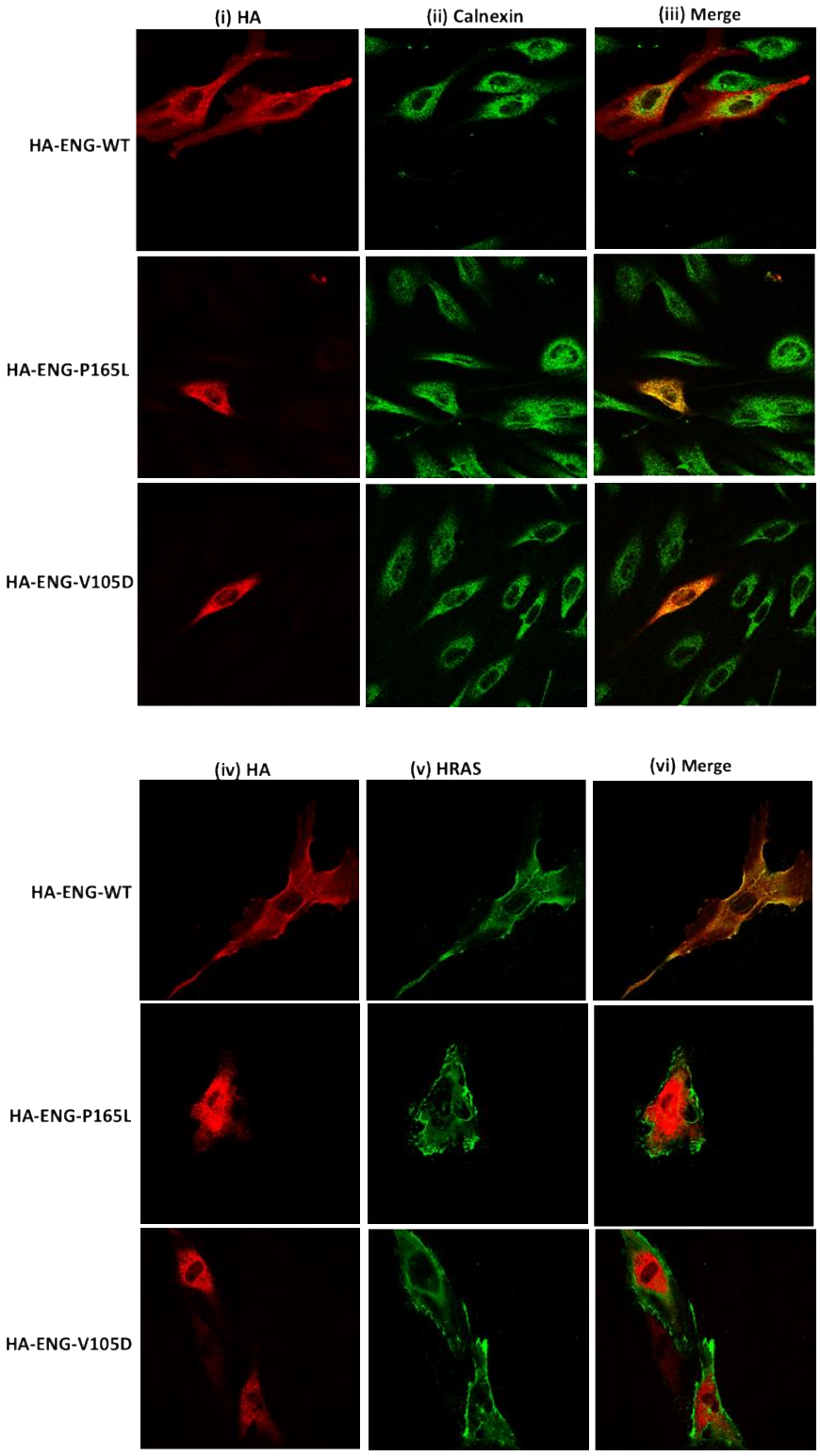

Figure 13: Subcellular localization of WT endoglin and mutant variants P165L and V105D in Telo-HAEC cell line

Immunofluorescence images investigating colocalization of HA-tagged WT endoglin and mutant variants P165L &V105D (Red, panels i and iv)) with ER marker (Calnexin, green, panel (ii)) and plasma membrane marker GFP-HRAS (green, panel v). Images were taken using fluorescence confocal microscope as described in the methods chapter. Nikon confocal microscope was used for image capture at (100X).

3.3 Investigation of the Cellular Half-Life and the Degradation Route(s) of Endoglin Wild-Type (WT) and Some ER-Retained Mutant Variants

3.3.1 The P165L and V105D Endoglin Variants are Highly Stable in Vivo Compared to the Wild Type Protein, but they do not form Aggregates

To analyze the kinetic of endoglin protein variants stability and degradation *in vivo*, cycloheximide chase experiments were carried out on HEK293 cells stably expressing WT endoglin and the two mutant variants P165L and V105D. Cycloheximide is a translation elongation inhibitor that blocks global protein synthesis and hence protein half-life can be determined. Stably transfected HEK293 cells were treated with cycloheximide and cell were harvested, and lysates prepared at 0-, 2-, 4-, 8-, 16- and 24-hours intervals and analyzed by Western blotting (Figure 14A, B and C). In the immunoblots, drastic decline in ENG-WT level was observed over the early time points of the chase experiment (Figure 14A). At 0 hours, WT-endoglin shows on the blot as two band, a higher band at 90KDa representing the fully glycosylated mature form (M), and a lower band at 80KDa representing the precursor form (P) in the ER. Around 2 hour from the start of the chase experiment, WT-endoglin had lost over 50% of its initial total protein (Figure 14A). The line graph, depicting the densitometric analysis of the immunoblots of cycloheximide chase assays (n=4), illustrates that WT endoglin has a short half-life of less than two hours (Figure 14D).

On the other hand, ER-retained variant P165L showing on the blot as a single precursor band at 80KDa (Figure 14B) remained relatively more stable through the 24 hour chase time. It was observed that after 2 hours, the level of P165L dropped from 100% to 70%, which is very likely due to the effect of cycloheximide inhibiting the translation of new proteins (Figure 14B). For the rest of the chase time (24hours), P165L maintained its stability around 75% (Figure 17D). Similarly, ER-retained V105D was revealed on the blot as a single precursor band at 80KDa (Figure 14D). At 8 hours, the level of P165L dropped to around 60%, followed by a slight increase to 70% towards the end of the chase at 24 hours (Figure 14D), was not statistically significant

Overall, a significant difference was detected between WT-endoglin and both variants P165L and V105D over the course of the chase period (Figure 14D). Both mutant

variants retained around 70% of their initial protein band intensity until the end of the 24-hour chase period, while WT-endoglin reached its half-life (50% protein level) in less than 2hours.

Because of this prolonged stability of the two mutants, we tested if they are forming aggregates inside the cells. To test for that, we carried out a Triton X-100 solubility assay which revealed that both endoglin WT and the two mutant variants P165L and V105D are soluble in the non-ionic detergent (Triton X-100) and hence are not forming detergent insoluble aggregates (Figure 14E). This was evidenced by the lack of any traces of endoglin in the pelleted fraction (P) of lysates that are likely to comprise detergent insoluble protein aggregates.

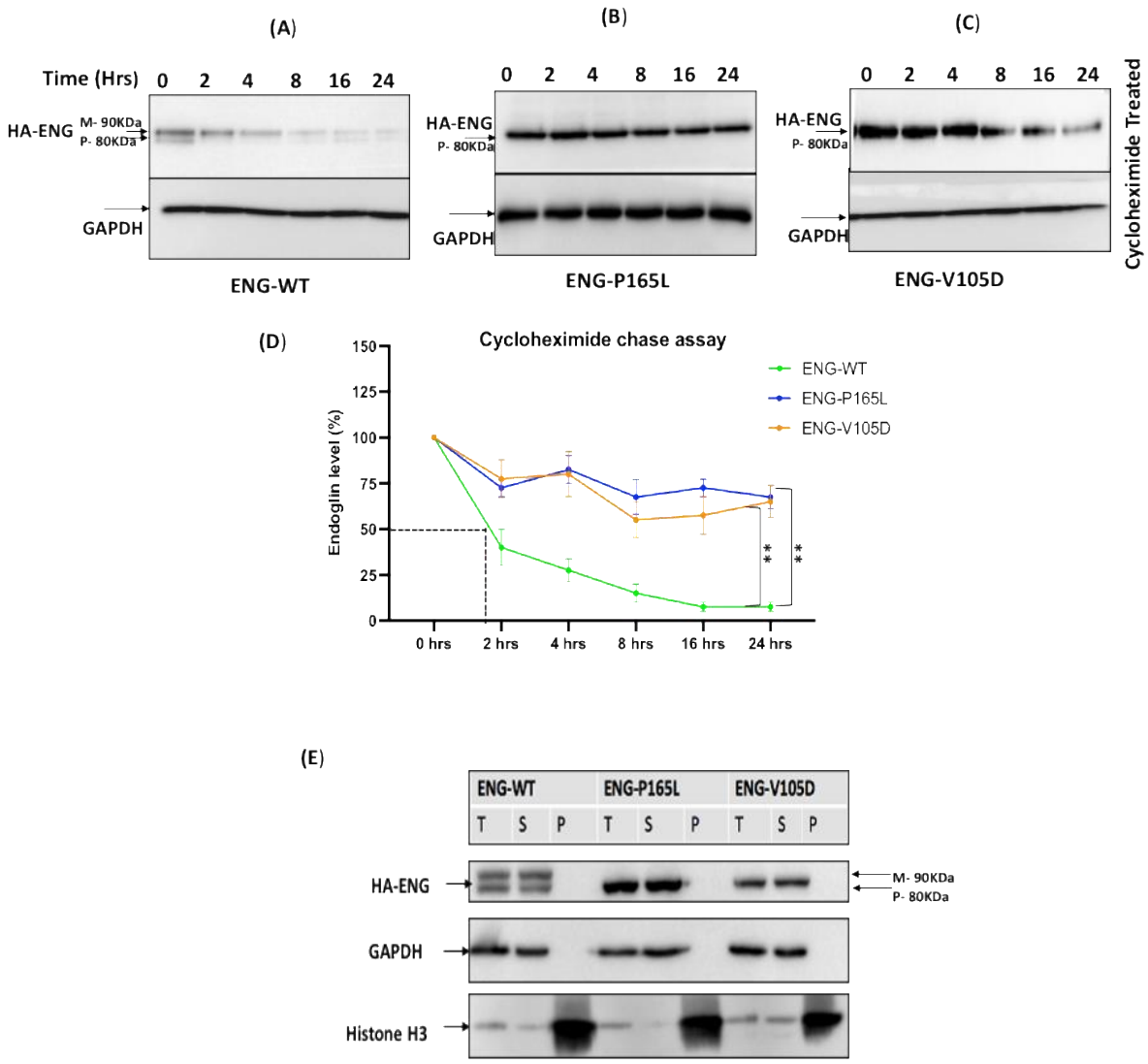

Figure 14: Cycloheximide (CHX) chase assay for stably transfected HEK293 endoglin WT and the two mutant variants P165L and V105D

(A) HEK293 stably expressing WT- endoglin shown on the blot by a Mature band (M) at 90KDa and precursor band (P) at 80 KDa treated with Cycloheximide (100 µg/ml) for the time points 0, 2, 4, 8, 16, 24 hours. (B) HEK293 stably expressing P165L- endoglin shown on the blot as a precursor band (P) treated with Cycloheximide (100 µg/ml) for the time points 0, 2, 4, 8, 16, 24 hours. (C) HEK293 stably expressing V105D-endoglin shown on the blot by a precursor band (P) treated with Cycloheximide (100 µg/ml) for the time points 0, 2, 4, 8, 16, 24 hours.(D) Line graph for endoglin Cycloheximide chase assay represents mean densities of WT endoglin and mutants relative to untreated at 0 hour, normalized with loading control (GAPDH). (E) Western blot analysis of endoglin WT and mutant variants' aggregation state. Harvested cells were lysed in 1%Triton lysis buffer (TBSt) followed by centrifugation at 20,000g at 4ºC. Supernatant (S), Pellet (P) and Total Lysate (T) were resolved in SDS-PAGE and then immunoblotted against HA-ENG. Histone H3 was used as a marker for P fraction and GAPDH for the T and S fractions. Error bars represent SEM from 4 independent experiments (n =4). Statistical significance between two groups was assessed using non-parametric Mann-Whitney test (GraphPad prism); (**) $P \leq 0.001$.

3.3.2 The P165L and V105D Variants Show Distinct Degradation Pathway Compared to Wild Type

HEK293 cells stably transfected with endoglin WT, P165L and V105D were treated individually with Bafilomycin, Eeyarestatin I, kifunensine, Epoximycin and MG132 for 24 hours then harvested and cell lysates were prepared and used for western blotting analysis to examine their stability under these treatments. Immunoblots generated are presented in Figure (15A, B and C) and then have been analyzed by densitometric analysis and illustrated in bar graph (Figure 15D) depicting accumulation levels of endoglin relative to DMSO treated cells (control). These results show significant accumulation of WT endoglin (over 4 folds) compared to DMSO only treated cells, when incubated with the lysosomal inhibitor Bafilomycin (Figure 15A and D). On the other hand, both variants P165L and V105D have shown a slight accumulation (~1.8 folds), when treated with Bafilomycin (Figure 15B, C and D). Treatment with the proteasomal inhibitors (MG132) have also resulted in significant accumulation of WT endoglin (~4.5 folds), however treatment with the other proteasomal inhibitor (Epoximycin) resulted in non-significant accumulation level (Figure 15A and D). MG132 and Epoximycin variably blocks the proteasomal pathway, but they have different chemical structures and mechanisms of action. MG132 is a peptide aldehyde proteasome irreversible inhibitor, while epoxomicin can reversibly inhibit proteolytic activity of the proteasome (Bo Kim et al., 2005). Therefore, it is possible that MG132 confer a more potent effect on WT endoglin compared to Epoximycin. This pattern of inhibition was also observed in a previous study conducted by our group (Kizhakkedath et al., 2019). These results suggest that WT endoglin undergoes both proteasomal and lysosomal degradations. It is very likely that the lysosomal pathway is responsible for the degradation process of mature endoglin receptor localized at the plasma membrane. It is very important to note here that 12-15% of newly synthesized proteins are also scrutinized through the ERQC mechanism, and temporarily misfolded or damaged proteins are degraded through the proteasomal pathway, which may explain the activated proteasomal degradation pathway for WT endoglin (Sun & Brodsky, 2019).

Unlike WT endoglin, both endoglin variant P165L and V105D have shown significant accumulation level when treated with Epoximycin (2.5 and 4.5 folds), respectively

(Figure 15B, C and D). Furthermore, treatment with MG132 have also resulted in significantly higher level of accumulation for both endoglin variants P165L and V105D (~5. 2 and 5.5 folds), respectively (Figure 15B, C and D). These findings further support the notion that MG132 demonstrates greater potency in inhibiting the proteasome compared to Epoximycin.

It is essential to highlight that not all forms of proteasomal degradation occur via the ERAD pathway. The proteasome is a large protein complex responsible for degrading proteins in the cell, and it participates in multiple degradation pathways including ERAD (Zhao et al., 2022). Hence, it was crucial to refine our study by employing precise proteasomal inhibitors that specifically target the ERAD machinery.

ERAD inhibition was achieved through the small molecules kifunensine (Kif) and Eeyarestatin I (EerI). EerI inhibits the deubiquitination mechanism associated with p97/VCP that facilitates the efficient recycling of ERAD substrates through the proteasomal pore (Stevenson et al., 2016). Treatment of WT-endoglin- expressing cells with EerI non-significant change in the WT endoglin expression level (Figure 15A). However, it was intriguing that the level of endoglin mutant P165L has significantly dropped by 50% after EerI treatment. The capability of Eer1 to produce a cytotoxic effect if incubation time is prolonged has been reported (Kizhakkedath et al., 2019; Liu & Ye, 2011). This effect has actually been utilized in anticancer therapy, as the disruption of ubiquitin homeostasis may trigger apoptotic death, hence it may explain the observed dramatic decline in endoglin expression (Liu & Ye, 2011). The toxicity of EerI can depend on several factors, including the cell type, concentration of EerI used, duration of exposure, and experimental conditions. In this experiment duration and concentration of Eer1 were optimized for minimal cellular death, however similar results were obtained. Similarly, treatment of HEK293 cells expressing mutant variant V105D, with EerI has not produced an accumulative effect on the protein level (Figure 15C & D). It has also been observed that EerI treatment has affected the mobility of both endoglin variants on the gel, which is represented by a high molecular weight smearing pattern on the immunoblots (Figure 15B and C), which most probably correspond to polyubiquitinated form of mutant variants P165L and V105D. This could potentially account for the observed decrease in endoglin levels across all three blots, corresponding

to both cells expressing the WT and mutant variants (Figure 15B, C and D). In addition, in a recent study, we have demonstrated that mutant variants P165L and V105D show accumulation of higher molecular weight ubiquitinated forms after MG132 treatment (Gariballa et al., 2022). These results further emphasize the role of the ubiquitin proteasomal pathway in the degradation of endoglin variants associated with HHT1.

Treatment with Kif, a potent inhibitor of mannosidase I enzyme, has not affected the level of either WT endoglin or the two mutant variants. Kif mainly acts on the mannosidase I enzyme, which plays a role in the recognition process of misfolded glycoproteins (Figure 10). Although inhibiting mannosidase I enzyme can interfere with the initial stages of ERAD, it might not completely obstruct all aspects of the ERAD pathway (Wang et al., 2011). Other elements of the ERAD machinery could still be functional, potentially reducing the effectiveness and selectivity of kifunensin as a complete ERAD inhibitor.

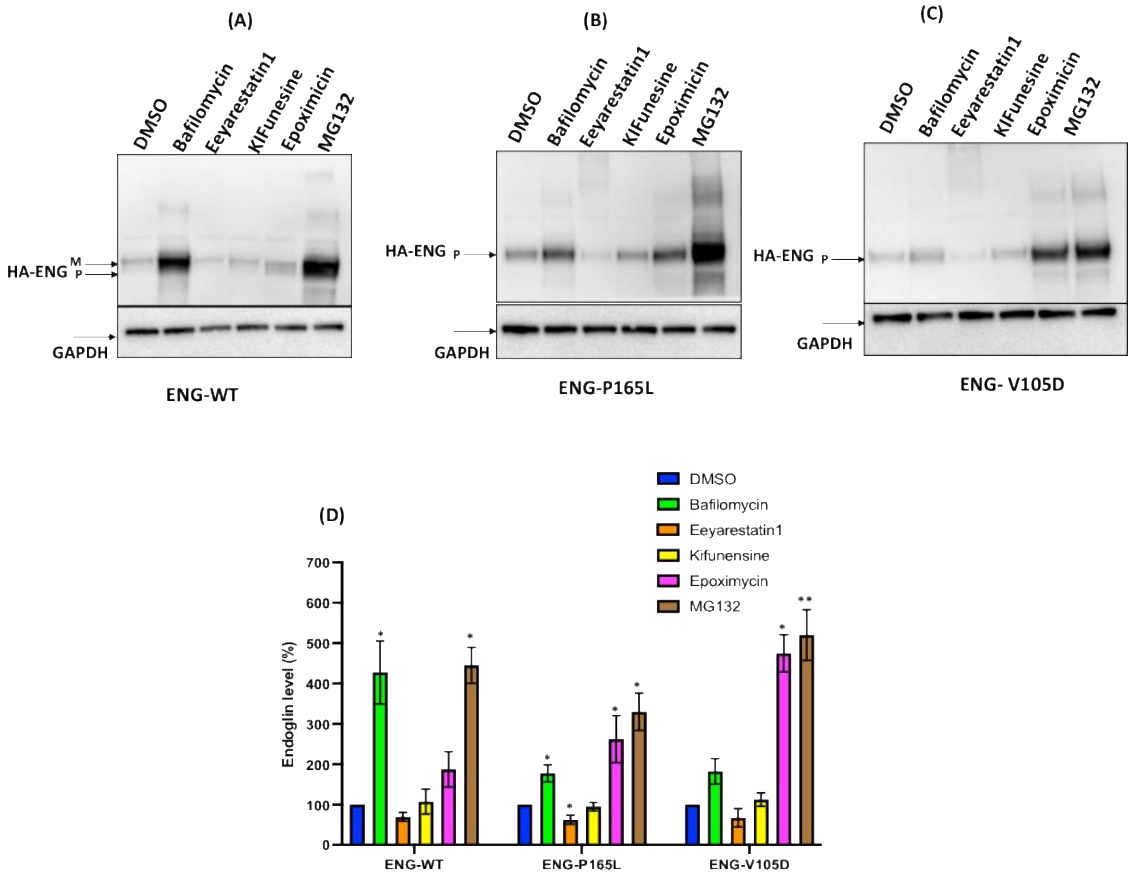

Figure 15: Degradation pathway of WT and mutant endoglin P165L and V105D

(A) Stable HEK293 cell lines harboring the HA-tagged ENG-WT were treated with Bafilomycin (200nM) (lysosomal inhibitor, Eeyrestatin1 (5μM) and Kifunensine (50nM) (ERAD inhibitors), MG132 (10μM) and Epoximycin (100nM) (proteasomal inhibitors). WT endoglin was shown on the blot as a mature band (M) at 90 KDa and precursor band (P) at 80 KDa, when probed with an anti-HA antibody. (B) Stable HEK293 cell lines harboring HA-tagged mutant variants p.165L with the same inhibitors as stated above. Variant P165L was shown on the blot as a precursor band (P) at 80 KDa, when probed with an anti-HA antibody. (C) Stable HEK293 cell lines harboring HA-tagged mutant variants p.V105D with the same inhibitors as stated above. Variant V105D was shown on the blot as a precursor band (P) at 80 KDa, when probed with an anti-HA antibody. Note: Each of these inhibitors have a unique mechanisms of action and each is likely to elicit a unique effect on the endoglin protein level. GAPDH was used as a loading control .(D) Bar graphs representing mean densities of WT endoglin and mutants normalized with GAPDH. endoglin level was expressed in (%) relative to DMSO treated (control). Bars represent SEM from 4 different Experiment (n=4). Statistical significance for each treatment relative to control was assessed using non-parametric Mann-Whitney test (GraphPad prism); (*) $p \leq 0.05$; (**); $p \leq 0.01$. Abbreviations: (M); Mature endoglin at 90KDa, (P); precursor endoglin at 80KDa.

3.4 Analysis of the Stability of the Mutant Variants in HEK293 Cell Lines Deficient in HRD1 E3 Ubiquitin Ligase, a Major Component of ERAD Retro-Translocon channel

In the previous section, We have demonstrated that endoglin mutant variants P165L and V105D are predominantly degraded through the proteasomal pathway, which may suggest the implication of the ERAD machinery in the degradation of these mutant proteins. However, specific inhibition of the ERAD mechanism using Kif and Eer1 small molecules has not produced a pronounced stabilizing effect on endoglin mutant variants P165L and V105D. So, here we aim to further investigate the implication of ERAD in the degradation of these mutant variants using a genome editing technique that can target key components of the ERAD machinery. In order to achieve that, we generated HEK293 knock out cell lines deficient in HRD1 E3 ubiquitin ligase, one of the major players in ERAD, using CRISPR Cas9 gene editing technique (Figure 11). As described in the methods section, six single-cell clones of HEK293 deficient in HRD1 E3 ubiquitin ligase were generated.

3.4.1 Generation of HEK293 HRD1-KO Clones Validated by Western Blotting and Sanger Sequencing

Gene knockout has been validated at the DNA level using sanger sequencing. At the protein level, total loss of expression was confirmed using Western blotting analysis (Figure 16). HEK293 clone carrying a CRISPR Cas9 scrambled control was generated as a negative control. All six generated knockout clones were carried forward for further

validation using sanger sequencing analysis. Primers were designed to amplify the 5 and 3 prime end sequence at the doner's integration site (Figure 17), taking into account that donor integration can be in forward or reverse orientation. Figure 17B and D shows sequence alignment of HRD1 KO clones G1H23 (KO 1) and G1H27 (KO 2) against WT sequence using Clustal Omega software to confirm the site of donor integration at the target sequence of the gRNA. Highlighted in the sequencing chromatograms the exact position where insertion mutations occurred during DNA repair as a result of DNA double strand breakage by the Cas9 enzyme followed by donor integration (Figure 17A and C). In both clones bi-allelic DNA perturbation occurred, and the donor was inserted at the target sequence of gRNA1.

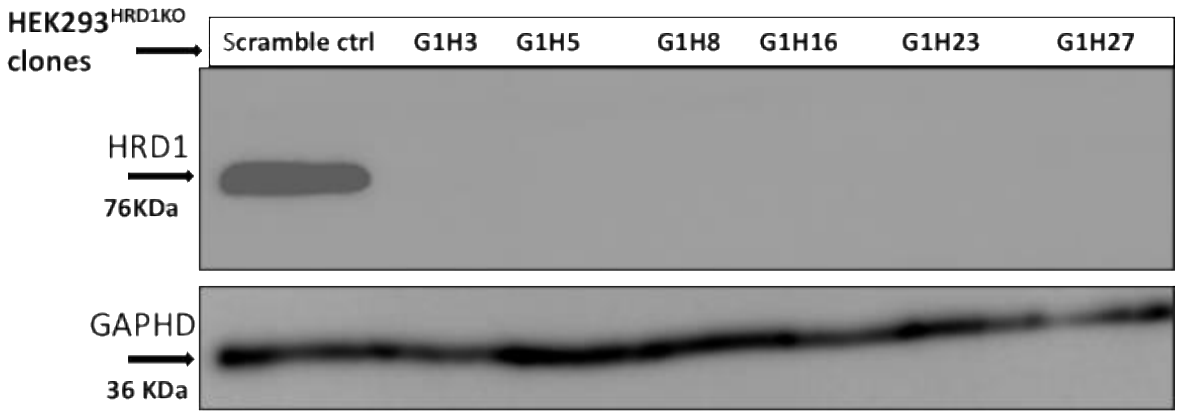

Figure 16: Validation of the generation of 6 HEK293-HRD1 knockout clones

Western blot image shows loss of HRD1 expression in the 6 HEK293 $^{HRD1\ Ko}$ generated clones (G1H3, G1H5, G1H8, G1H16, G1H23 AND G1H27) when probed with an anti-HRD1 antibody. On the other hand, HEK293-scrambled negative control shows strong expression of HRD1 represented by a band at position at position 76 KDa.

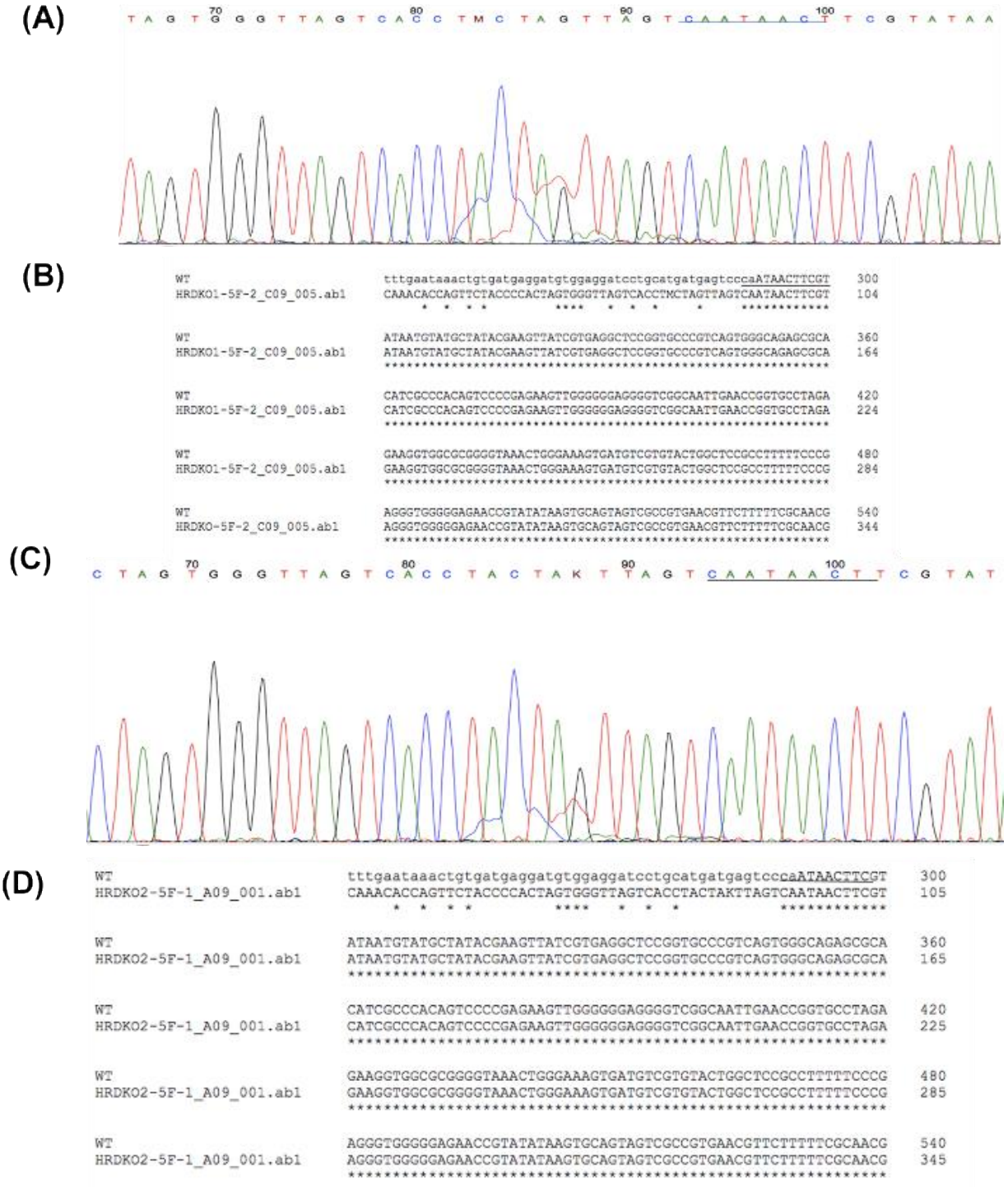

Figure 17: Sanger sequencing validation of HRD1 knockout in HEK293 cells

(A and C) show Clustal Omega sequence alignment of WT HRD1 with PCR amplification product of KO1(G1H23) and KO2 (G1H27) clones, respectively, at the target site of donor integration. (B and D) represent the sequencing chromatogram of the aligned sequence of both KO1 and KO2 at the site of donor integration. The underlined sequences represent the site of donor integration preceded by a stretch of insertion mutations.

3.4.2 Significant Accumulation of P165L and V105D Endoglin Variants as a Result of HRD1 Deficiency

In order to investigate the role of such evolutionary translocon channel in the degradation pathway of endoglin WT and mutant variants P165L and V105D, we have utilized CRISPR-Cas9 gene editing technology to create HEK293 cell lines deficient in HRD1 E3 Ubiquitin ligase. Clonal HEK293 $^{HRD1\text{-}KO}$ cells were individually transiently transfected with plasmid vectors harboring WT endoglin and mutant variants P165L and V105D (Figure 18). For the purpose of transfection efficiency control, cells were co-transfected with RFP plasmid vector. Immunoblotting results have shown that endoglin variants P165L and V105D have significant protein accumulation in the two HEK293$^{HRD1\text{-}KO}$ cell lines (HRD1 KO 1# and HRD1 KO 2#) compared to that observed in HEK293^{Sc} (scrambled control) (Figure 18A and B). These findings emphasize the essential role of HRD1 E3 ubiquitin ligase in the degradation pathway of misfolded endoglin variants that further implicates the role of ERAD machinery in the pathology of HHT1. On the other hand, WT endoglin has shown insignificant accumulation level in HEK293 $^{HRD1\ KO}$ cells, which could indicate that proteasomal degradation of WT endoglin does not process significantly through the HRD1/SEL1 translocon channel.

These findings confirm our prediction that misfolded endoglin variants trapped in the ER lumen are likely to be recognized as ERAD substrates and subjected to retro-translocation through the HRD1/SEL1L translocon channel to be degraded in the cytosol through the proteasomal machinery (Figures 9 &10).

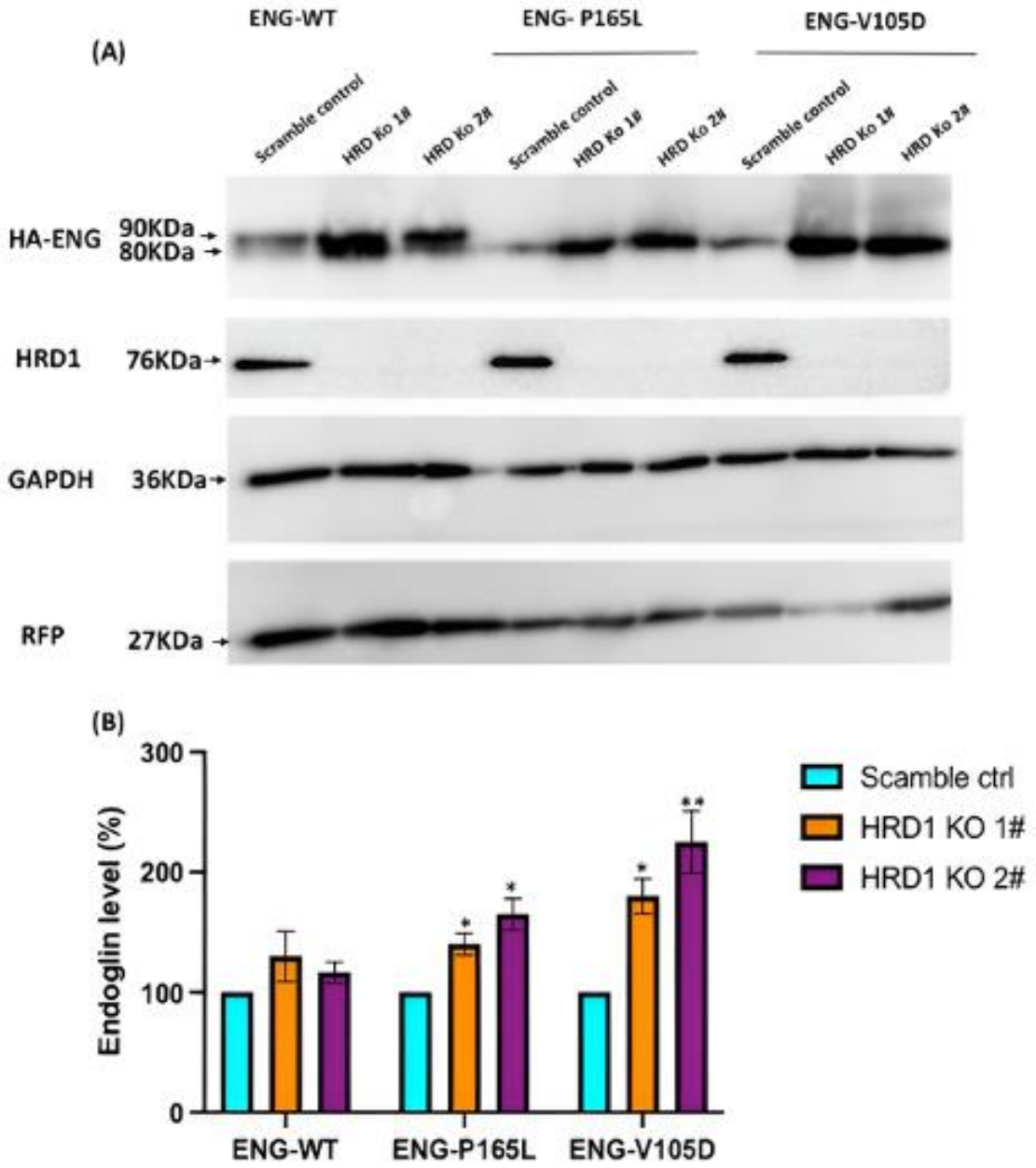

Figure 18:Endoglin WT and mutant variants accumulation level in HEK293 $^{HRD1\text{-}KO}$ cells

(A) Generated HRD1-Knockouts (1# and 2#) were transiently transfected with pcDNA3.0 plasmid vectors harboring WT endoglin and mutant variants (P165L and V105D). Total cell lysate was analyzed by immunoblotting against HA-ENG, RFP, GAPDH and HRD1 (B) Bar graphs representing mean densities of WT endoglin and mutants normalized with GAPDH (loading control) and RFP (For transfection efficiency control). endoglin level was expressed in (%) relative to (scramble control). Bars represent SEM from 4 Experiment repeats (n=4). Statistical significance for endoglin accumulation level in each cell line (relative to scramble control) was assessed using non-parametric Mann-Whitney test (GraphPad prism (*) $p \leq 0.05$; (**); $p \leq 0.01$

3.5 Explore the Potential for Manipulating the Folding and Trafficking of Endoglin Mutants using Chemical Chaperones

3.5.1 Glycerol and 4-PBA Chemical Chaperones Failed to Correct P165L and V105D Mutants' Defective Trafficking

Our observation in the previous sections have shown that accumulation of mutant variants P165L and V105D via blocking the proteasomal degradation pathway does not enhance their trafficking to the plasma membrane, as they continued to largely show on the Western blot as a precursor protein (Figure 15B &C). Therefore, in this section, in an attempt to rescue the misfolding and trafficking defects of variants P165L and V105D, we have utilized chemical chaperones glycerol and 4-PBA. HEK293 cells stably expressing WT endoglin and the two variants have been incubated with 5% glycerol or 2.5mM of Sodium Phenylbutirate 4-PBA. Live cells were collected and immunostained with endoglin conformation specific antibody P3D1, as described in the methods section. The monoclonal antibody P3D1 recognize the N-terminal region of 204 amino acids encoded by exons 1–5. The antibody can only recognize plasma membrane localized endoglin. A FITC conjugated secondary antibody was used to visualize immunostained endoglin protein using FACS technique. FACS analysis of untreated HEK293 cells expressing WT endoglin have shown ~ 48% of positively stained cells for mature endoglin located at plasma membrane (Figure 19A & B). In contrast, cells expressing the V105D variant exhibited minimal effects, whereas cells expressing the P165L variant showed no detectable positive signal. These results are in accordance with all our previous Western blotting data that shows mutant variant P165L and V105D as a single band at 80KDa representing endoglin precursor located in the ER.

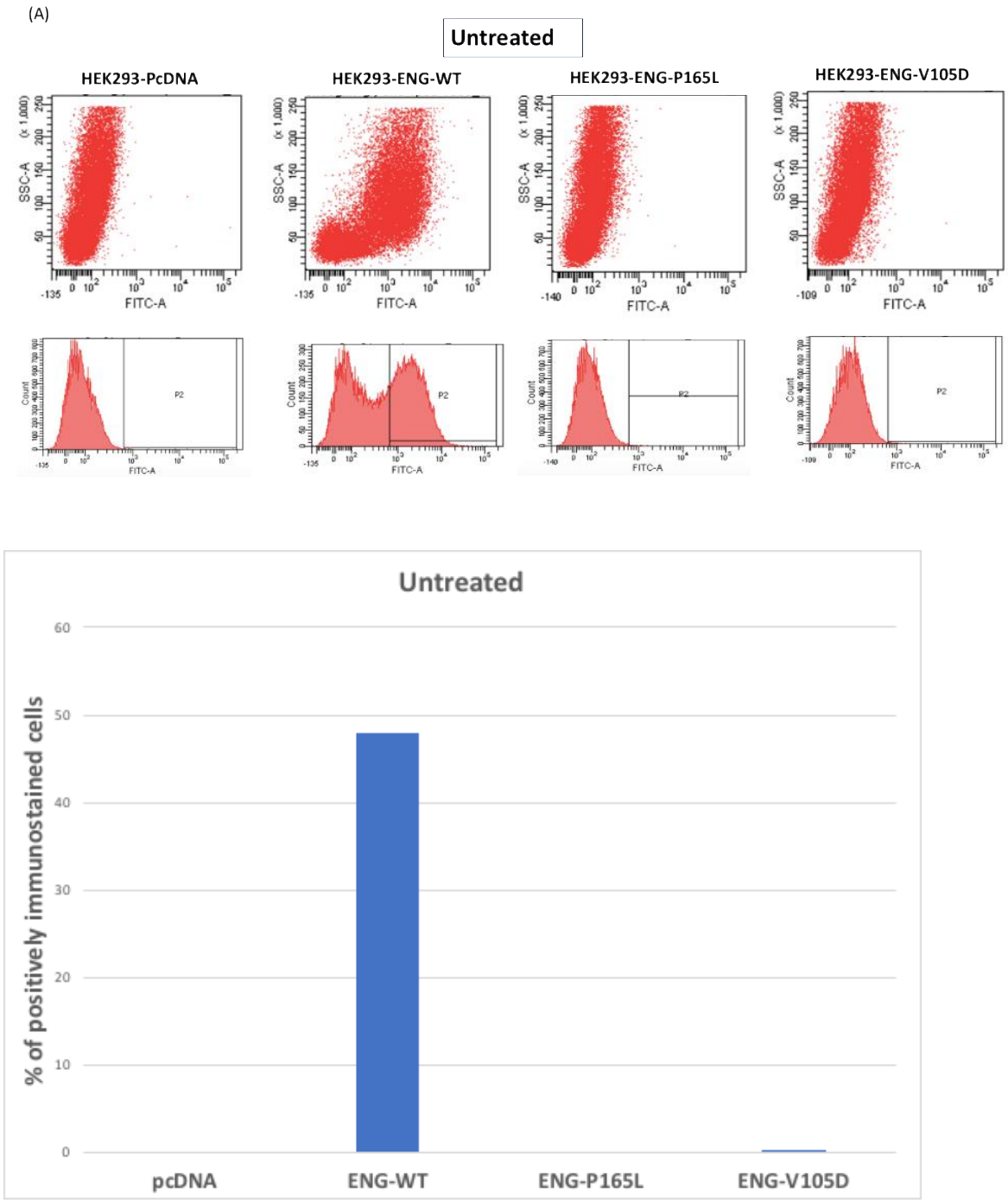

Figure 19: HEK293 cells immunostained with P3D1 antibody confirms subcellular localization of WT endoglin and mutants P165L and V105D

(A) FACS plots of untreated HEK293 cells stably transfected with WT endoglin and mutant variants P165L and V105D. Cells were immunostained with P3D1 configuration-specific endoglin antibody. Cells harboring an empty vector were used as a negative control. (B) Bar graphs representing the percentage of positively stained cells.

Similarly, HEK293 cell lines stably expressing WT endoglin and mutant variants P165L and V105D were treated with 5% glycerol, which serves as a chaperone, supporting the accurate folding of protein structure (Diamant et al., 2001). By establishing hydrogen bonds with hydrophobic regions exposed on proteins, glycerol prevents protein aggregation and facilitates proper folding. Moreover, it safeguards protein stability by minimizing denaturation and inhibiting the formation of non-functional protein aggregates (Diamant et al., 2001). FACS analysis of HEK293 cells expressing WT endoglin have reported ~ 27% of positively stained cells for mature endoglin located at plasma membrane (Figure 20A & B). On the other hand, variants P165L and V105D have shown minimal increase in number of positively stained cells for mature endoglin, when compared with cells transfected with an empty vector (Figure 20A & B).

In a similar experimental setup, 4-PBA, recognized for its role as a chemical chaperone in aiding protein folding and alleviating ER stress, was employed. 4-PBA can help reduce the accumulation of misfolded proteins enhancing their folding and preventing their aggregation. Our FACS plots recorded minimal scatter of positively immunostained HEK293 cells expressing variants P165L and V105D when treated with 4-PBA chemical chaperone (Figure 21A & B).

It was however intriguing to observe that the percentage of recorded positively stained cells expressing WT endoglin dropped from 48%, when untreated, to 27% and 31% when treated with Glycerol and 4-PBA, respectively (Figures 19B, 20B & 21B). The significance of this decline could have been examined had the experiment been repeated. Nonetheless, the objective of this study was to observe whether these chemical chaperones could induce a rescue effect on the mutant variants.

In conclusion, chemical chaperones 4-PBA and glycerol don’t appear to positively impact the trafficking of mutants P165L and V105D from the ER to the plasma membrane. This aspect of the study warrants further investigation, employing alternative chemical chaperones that have shown rescue benefits in other mutant proteins.

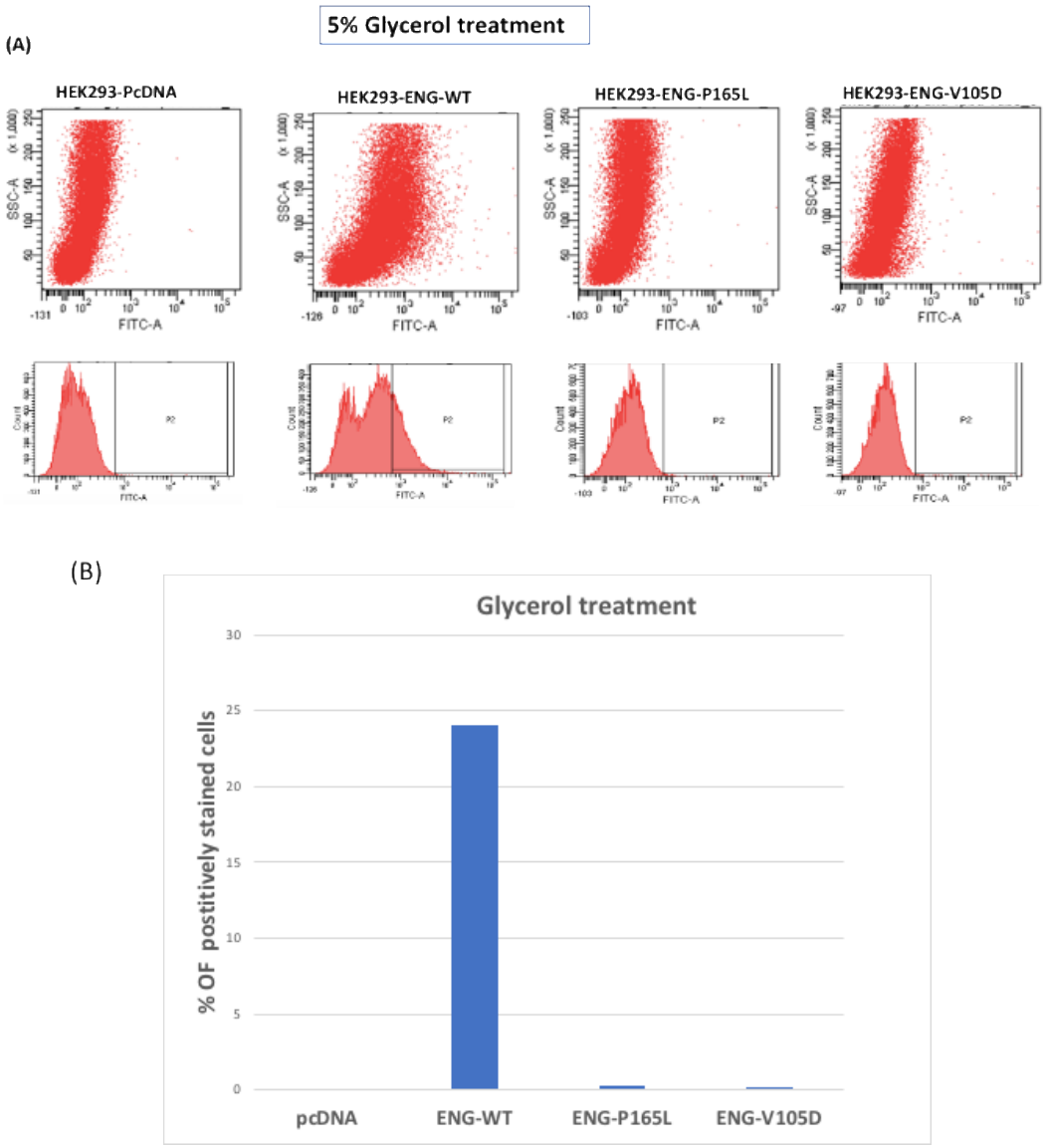

Figure 20: Glycerol treatment has not enhanced the trafficking of variants P165L and V105D

(A) FACS plots of HEK293 cells stably transfected with WT endoglin and mutant variants P165L and V105D treated with Glycerol. Cells were immunostained with P3D1 configuration-specific endoglin antibody. Cells harboring an empty vector were used as a negative control. (B) Bar graphs representing the percentage of positively stained cells.

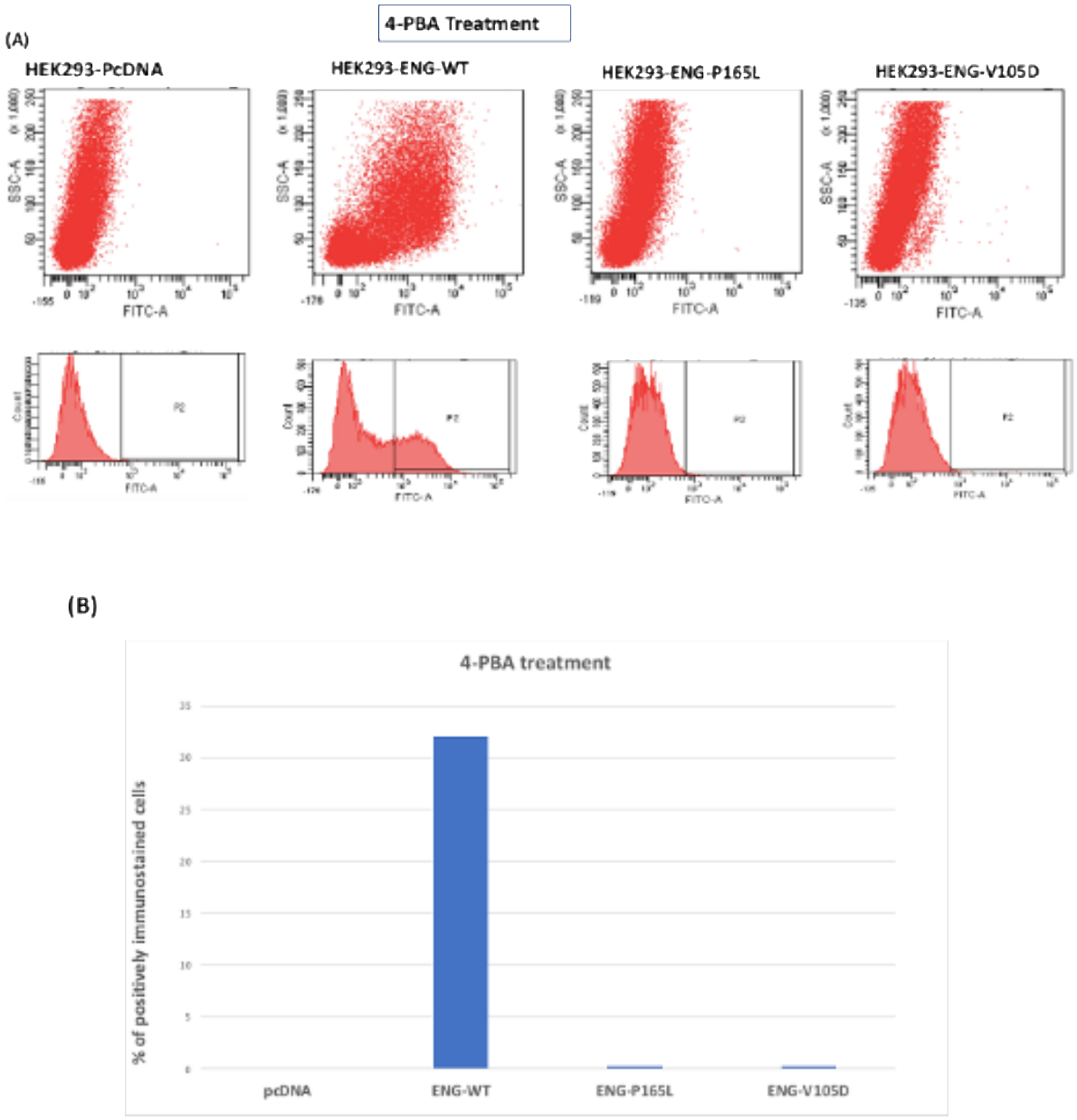

Figure 21: 4-PBA treatment has not enhanced the trafficking of variants P165L and V105D

(A) FACS plots of HEK293 cells stably transfected with WT endoglin and mutant variants P165L and V105D treated with 4-PBA chemical chaperone. Cells were immunostained with P3D1 configuration-specific endoglin antibody. Cells harboring an empty vector were used as a negative control. (B) Bar graphs representing the percentage of positively stained cells.

3.6 Investigate the Possibility of a Dominant Negative Effects Exerted by ER-Retained Endoglin Variants on the WT Allele

3.6.1 The ER-Retained Mutant Variants P165L and V105D Interact with WT Endoglin Suggesting the Formation of Heterodimers

HHT1 is an autosomal dominant genetic disease, which means that one copy of the inherited gene is still expressing WT endoglin that accounts for 50% of the total protein expressed and is consequently expected to be functional. The mutant copy of the gene accounts for the remaining 50% of the protein expressed and therefore when lost due to the mutation the disease is said to be caused by haploinsufficiency. In the previous sections, I have demonstrated the subcellular trafficking defects and the degradation pathways of WT endoglin and mutant variants P165L and V105D in HEK293 cells that indigenously lacks the expression of endoglin. We were therefore able to track the biosynthesis, trafficking and degradation of the stably expressed WT endoglin and mutants without any interference from indigenous endoglin expression. However, because most HHT1 patients are heterozygous, we wanted to examine if some of the mutant alleles, when expressed, are interfering with the trafficking and function of the WT allele. Conversely, we also wanted to test if the WT allele can rescue the trafficking defect of some of the ER-retained mutant alleles when overexpressed. The justification for these assumptions is that endoglin is a homodimer and this dimerization occurs early within the secretory pathway, namely within the ER. In addition, we wanted to investigate if the ER-retained mutant variants P165L and V105D are exerting a dominant negative effect on the WT protein. Dominant negative effect occurs when the mutant variant interferes with the trafficking, stability or localization of the WT protein which may result in an overall decrease in the WT function as well. Heterodimerization between endoglin WT and mutant alleles is, therefore, a plausible scenario. Additionally, the formation of heterodimers with endoglin mutant variants that are trapped in the ER might affect the trafficking of wild-type endoglin and consequently impair its subcellular localization and function.

To test this possible interaction between WT and the mutant alleles, HEK293T cells stably expressing the HA-tagged WT endoglin and the mutant variants P165L and V105D were transiently transfected with pCMV6 vector harboring Myc-tagged WT

endoglin. Western blotting of lysates from these cells shows that both proteins are expressed as expected, with both WT-Myc and WT-HA endoglins expressed as two bands (mature and immature at ~90 and ~80 KDa). As shown in previous experiments, the two mutants are expressed as a single immature band at around 80KDa (Figure 22 Panel A). To test the interactions between the Myc and HA tagged proteins in this system, equal amounts of total cell lysates were immunoprecipitated with anti-HA agarose beads, followed by probing the Western blots of the immunoprecipitates with anti-Myc antibody as described in the Methods section. As expected, WT Myc-tagged endoglin has been co-immunoprecipitated with HA- tagged WT suggesting their dimerization. Crucially, both the fully mature and immature species were co-immunoprecipitated confirming that this dimerization occurs in the ER prior to maturation (Figure 22B, lane 2). Importantly, when we immunoprecipitated the HA-tagged P165L and V105D mutants, we could detect substantial amounts of the immature Myc-WT (Figure 22B lanes 3and 4). It is important to note that the mature form of WT-Myc was not present in these immunoprecipitates which confirms the interaction between WT and the two mutants, and their dimerization take place within the ER.

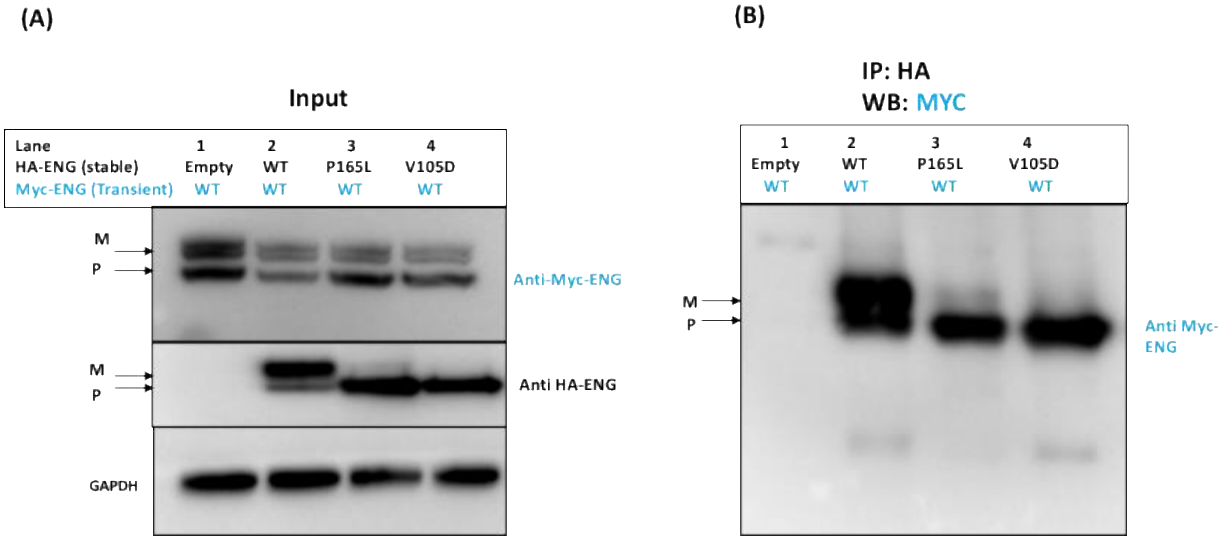

Figure 22: Myc-tagged WT endoglin interacts with stably transfected mutant variants P165L and V105D

Stably transfected HEK293 cells with WT-endoglin and variants P165L and V105D were transfected with Myc-Tagged WT endoglin. An empty vector was used for negative control. (A) Inputs were probed with anti-Myc, anti-HA and anti GAPDH. (B) Total cell lysates were immunoprecipitated with anti-HA agarose beads and probed with anti-Myc antibody. Abbreviations: (M) Mature endoglin, (P) Precursor endoglin

3.6.2 The ER-Retained Mutant Variants P165L and V105D Interfere with the Maturation of WT Endoglin Suggesting their Dominant Negative Effects

In the previous experiment HA-tagged endoglins were stably expressed and the Myc-tagged WT was transiently co-transfected into these cells and therefore the levels of expression of the two types of proteins are not equal and cannot be easily controlled. To exert some level of control over this situation, we transiently transfected the cells with equal amounts of DNA of the two vectors. Again, we agree that this might not guarantee that the required level of control on protein expression can be achieved with the required accuracy. In particular, we wanted to vary the amounts of the expressed mutant proteins to see if we can interfere with the level of Myc-tagged WT maturation. To test if we can reproduce the results on the interactions of the HA and Myc tagged proteins obtained in the previous section, we transfected HEK293T cells with a 1:1 mixture of the two DNA vectors. Western blotting analysis of the lysates from these cells after 48 hours of expression is shown in Figure 23A which indicates that both proteins are expressed and behave as shown previously, with the wild types appearing as two bands (mature and immature bands) and the mutants as a single immature band. Immunoprecipitating with Anti-HA antibodies and then Western blotting with anti-Myc antibodies is shown in panel B of figure 23. The results are exactly the same as with the previous experiment using the stably transfected cells in figure 22. The immature band of Myc-WT has precipitated with anti-HA antibodies when the ER-retained mutants are co-expressed (Figure 23B lanes 3 & 4). This confirms the interactions of WT and mutants, and the formation of heterodimers in the ER.

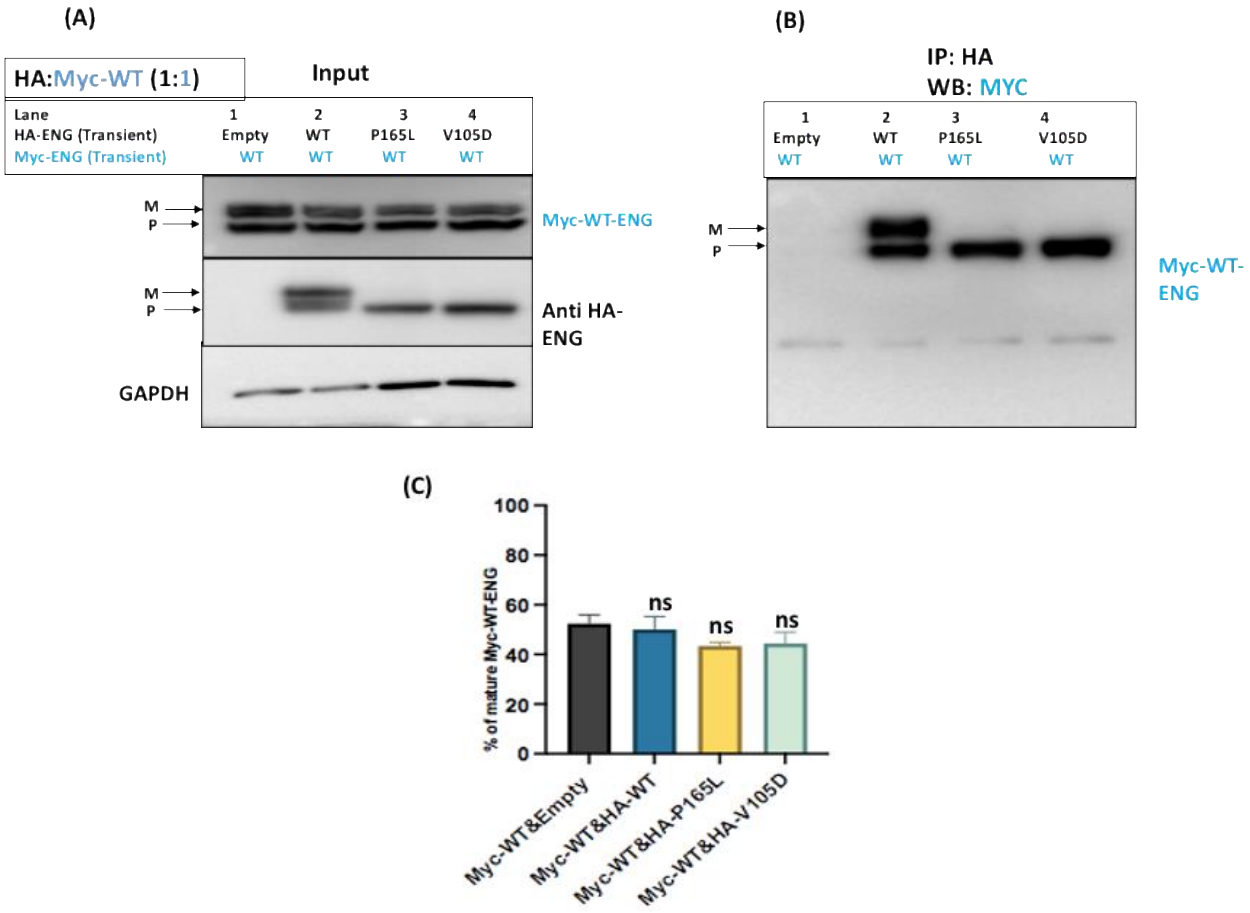

Figure 23: Myc-tagged WT endoglin interacts with transiently transfected mutant variants P165L and V105D without interfering with Myc-WT endoglin

HEK293 cells were co-transfected in a ratio of 1HA:1Myc with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants P165L and V105D. (A)Western blots of total cell lysates (inputs) were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-4). (B)Western blots showing HA immunoprecipitates probed with anti-Myc antibody (Lanes 5-8). (C) Bar graph representing mean densities of mature Myc-WT endoglin as a percentage of total protein expressed. Bars represent SEM from3 experiments (n=3). Statistical significance of percentage of mature Myc-WT endoglin co-transfected with HA-WT, P165L &V105D in a ratio of (4HA: 1Myc), relative to its expression alone (empty vector) was determined using unpaired t-test (GraphPad Prism), Abbreviations: (M) Mature endoglin, (P) Precursor endoglin, (ns) non-specific

Next, we examined if the level of Myc-WT maturation is influenced by the expression of the two ER-retained mutants. About 50% of the expressed Myc-WT is in the mature higher molecular weight band. Incidentally, this level is lower than what we observed for the HA tagged version, which might reflect the effects of the different tags. We quantified the level of maturation expressed when we transfected the cells with equal amounts of DNA and in this case the level of Myc-WT maturation dopped to around

40% when co-expressed with the two ER-retained mutants (Figure 23C). To test this observation further and to quantify it more accurately, we increased the ratio of the mutants DNA to WT from 1HA:1Myc to 4HA:1Myc. The Western blot analysis of this is shown in figure 24A. It was noticeable that the level of Myc-WT maturation (i.e. the proportion of the higher molecular weight mature band) is much lower when the mutants were co-expressed at 4:1 ratio (compare lane 1 and 2 with lanes 3 and 4, Figure 24A). We quantified the level of maturation and it was, 25% and 7% for P156L and V105D, respectively (Figure 24C). The interactions between the Myc and HA versions of the proteins are shown and presented in figure 24B which confirms that the ER-retained mutants interact and dimerize with the immature form of the Myc-WT-.

This data strongly suggest that the ER-retained mutants do indeed trap the WT in the ER and prevent its maturation significantly, thus confirming our suspicion of their dominant negative effects.

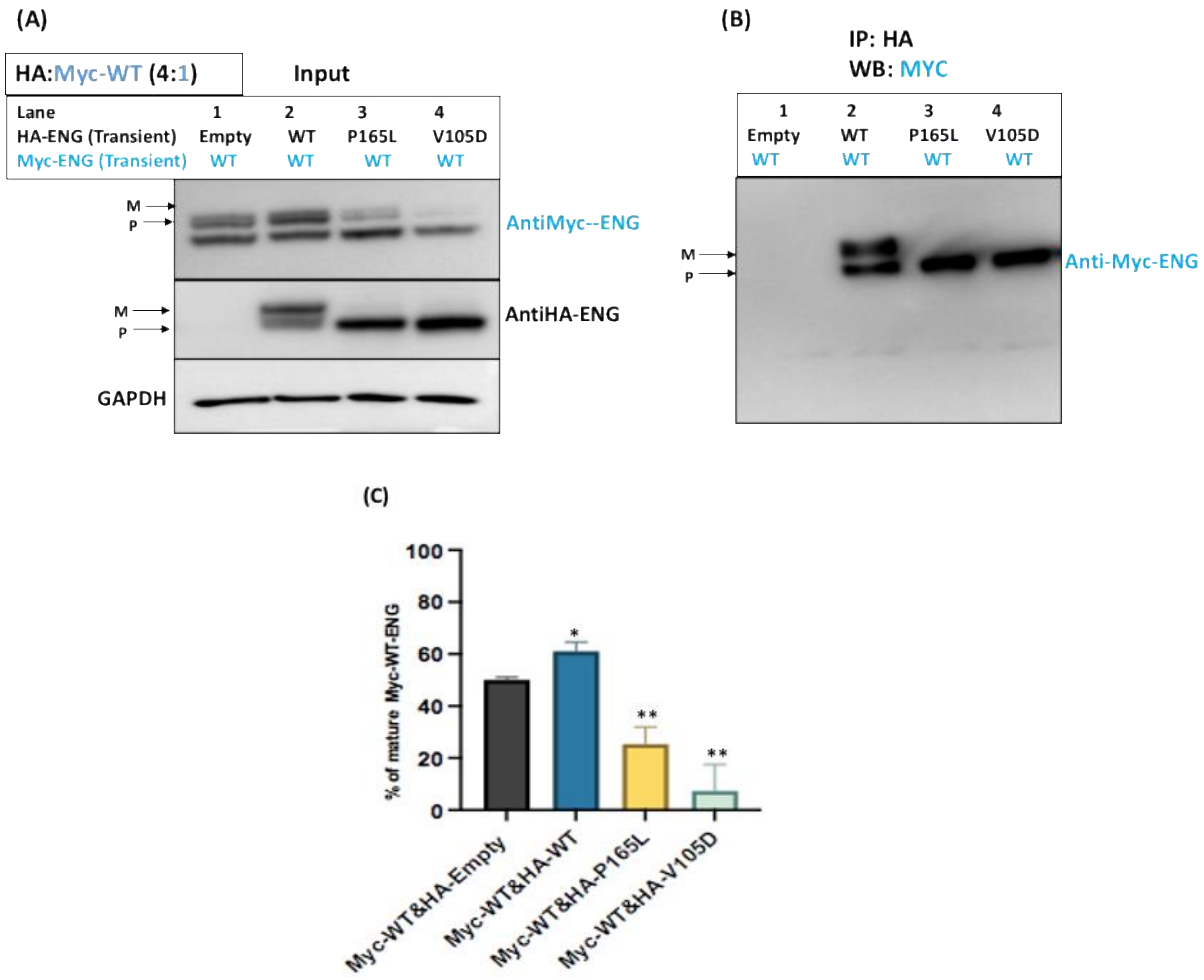

Figure 24: Formation of heterodimers between Myc-WT endoglin and variants P165L and V105D impairs the maturation of WT endoglin

HEK293 cells were co-transfected in a ratio of (4HA:1Myc) with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants P165L and V105D. (A) Western blots of total lysate (inputs) were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-4). (B) Western blots showing HA immunoprecipitates probed with anti-Myc antibody (Lanes 5-8). (C) Bar graph representing mean densities of mature Myc-WT endoglin as a percentage of total protein expressed. Bars represent SEM from3 experiments (n=3). Statistical significance of percentage of mature Myc-WT endoglin co-transfected with HA-WT, P165L &V105D in a ratio of (4HA: 1Myc), relative to its expression alone (empty vector) was determined using unpaired t-test (GraphPad Prism) (*) $p \leq 0.05$; (**); $p \leq 0.01$. Abbreviations: (M) Mature endoglin, (P) Precursor endoglin.

In order to further demonstrate the effects of those mutants on the maturation of Myc-WT endoglin, we investigated the subcellular localization of Myc-WT endoglin in the presence of excessive amounts of the ER-retained mutants P165L and V105D. Similar to the previous experiment, HEK293T cells were co-transfected with pCMV6 vector

harboring Myc-WT endoglin and pcDNA3.0 expressing P165L or V105D mutant variants. The transfection was carried out at a DNA ratio of (4HA: 1Myc). Cells were immunostained against the Myc tag representing Myc-WT endoglin (Figure 25 panel (i), red), plasma membrane marker Na^+K^+ ATPase (Panel (ii), green). Observation of the merged image (panel iii) shows clear intracellular localization of Myc-WT endoglin when co-transfected with either of the mutant variants (P165L and V105D). This was demonstrated by the sharp red immunostaining inside the cell with no apparent colocalization with the plasma membrane marker Na^+K^+ ATPase (Figure 25). On the other hand, co-transfection of Myc-WT endoglin with either the empty vector or HA-WT endoglin shows clear colocalization with the plasma membrane marker, as it is normally expected with WT endoglins (Figure 25).

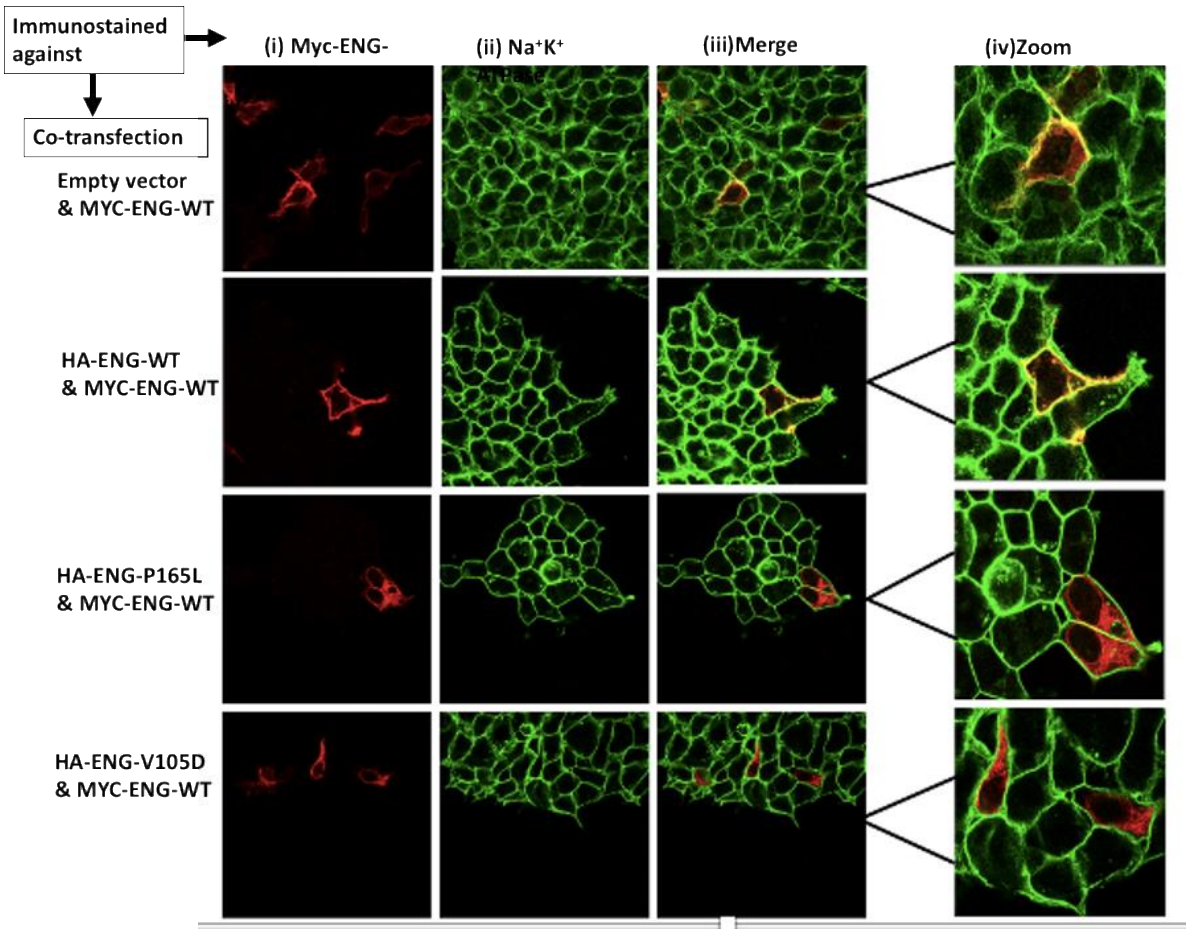

Figure 25: Intracellular localization of Myc-WT endoglin under the dominant negative effect of mutant variants P165L and V105D

Immunofluorescence images investigating intracellular Myc-WT endoglin Panel (i), red) co-transfected with WT endoglin and variants in a DNA ratio of (4HA:1Myc), relevant to plasma membrane marker Na^+K^+ATPase (Panel (ii) green). A merged and zoomed image of anti-Myc and anti-Na^+K^+ATPase represented in (iii) and (v), respectively. Images were taken using fluorescence confocal microscope at (100X).

In order to visualize the intracellular interaction between Myc-WT endoglin and ER-retained mutant variants P165L and V105D, cells were immunostained against the HA tag representing HA-WT endoglin and mutant variants P165L, V105D (Figure 26 Panel (i), red), Myc tag representing Myc-WT endoglin (Panel (ii), green) and Calnexin, ER marker, (panel (iii), blue). A merged image of the three is depicted in panel (iv). Similar to our earlier Western blot analysis, our results show that Myc-WT endoglin shows plasma membrane expression when co-expressed with an empty vector. In contrast, co-expression of Myc-WT endoglin with the ER retained mutant variants P165L and V105D in a ratio of (4HA: 1Myc) resulted in a clearly observed colocalization of Myc-

WT-endoglin with the ER calnexin marker and HA tagged mutant variants (Figure 26). On the other hand, Myc-WT endoglin shows plasma membrane localization when co-expressed with HA-WT endoglin, demonstrated by colocalization of the two WT endoglin proteins (Figure 26).

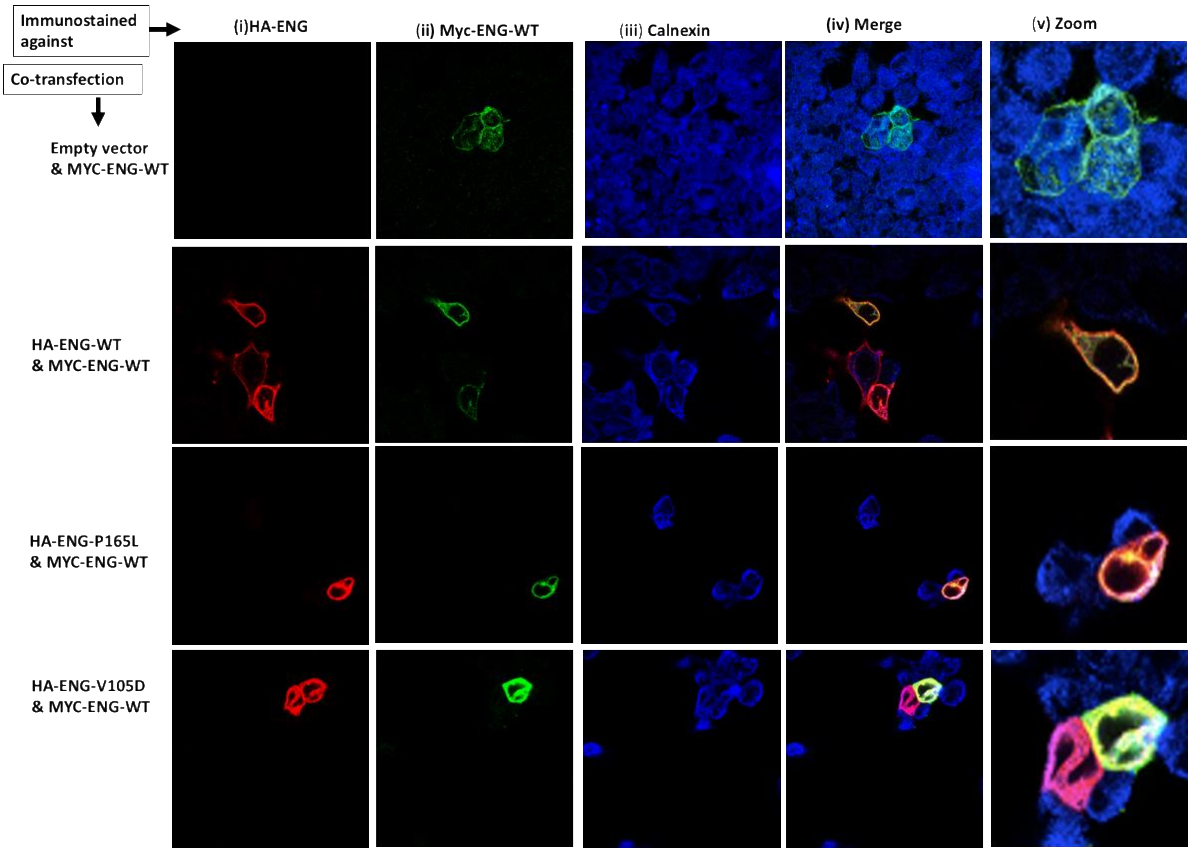

Figure 26: Myc-WT endoglin forms heterodimers with P165L and V105D form in the ER

HEK293T cells were co transfected in a ratio of 4HA: 1Myc with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants P165L and. Cells were immunostained against HA tag (Panel (i), red), Myc (Panel (ii), green) and Calnexin ER marker, (panel (iii), blue). A merged image of the three is depicted in panel (iv). (v) is a zoomed in image of (iv). (Nikon confocal microscope was used for image capture at (100X)

We have also investigated if excessive expression of Myc-WT could at least partially rescue the mutant variants from ER retention and allow them to traffic to the plasma membrane. We expressed a mixture of Myc-WT and mutants at 4:1 ratio (4Myc-WT:1HA-Mutants). Our results show that excessive overexpression of Myc-WT-endoglin does not positively impact the maturation of either of the two ER-retained

mutant variants P165L and V105D (Figure 27A lane 3 &4). This was noticeable on the blot as both variants were revealed as single precursor band when probed with ani-HA antibody.

WT proteins are sometimes potentially capable of rescuing ER- retained mutants through a process known as (complementation) (Cebotaru & Guggino, 2014). It occurs when the WT is capable of interacting with mutant protein and assisting its folding and trafficking to its functional destination. However, it is well known that not all mutant proteins can be rescued by the WT, as the nature of the protein and the type of mutation can greatly influence the degree to which "complementation" can occur.

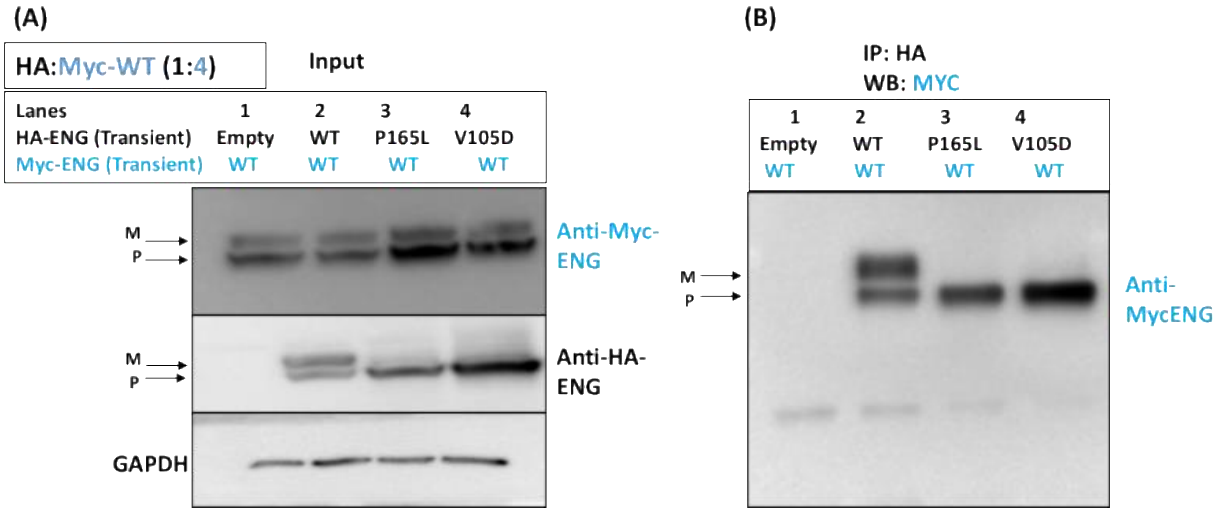

Figure 27: Excess expression of WT endoglin doesn't assist the trafficking out of the ER and maturation of mutant variants P165L and V105D

(A)HEK293T cells were co transfected in a ratio of (1HA:4Myc) with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants P165L and V105D. (B)Western blots showing HA immunoprecipitates probed with anti-Myc antibody (Lanes 5-8). Western blots of inputs were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-4). Abbreviations: (M) Mature endoglin, (P) Precursor endoglin.

3.6.3 Other ER-Retained Mutants Interfere with WT Maturation and Cause Dominant Negative Effects

We wanted to extend our investigation to test the potential dominant negative effects of other HHT1-disease causing variants that have been shown to cause endoglin ER retention (Ali et al., 2011). We therefore tested another three variants (L32R, C53R, I271N) within the ligand-binding Orphan domain and one variant (C363Y) within the Zona Pellucida-N domain, which is thought to play a key role in homo-dimerization of the endoglin co-receptor (Saito et al., 2017). All four missense mutant variants were confirmed to be retained in the ER by being present in the immature form; lower molecular weight only as illustrated in the Western blotting analysis (Figure 28A). ER-retention was also confirmed by immunofluorescence imaging, in which both mutants were shown to be co-localized with the ER marker calnexin (Figure 28B)

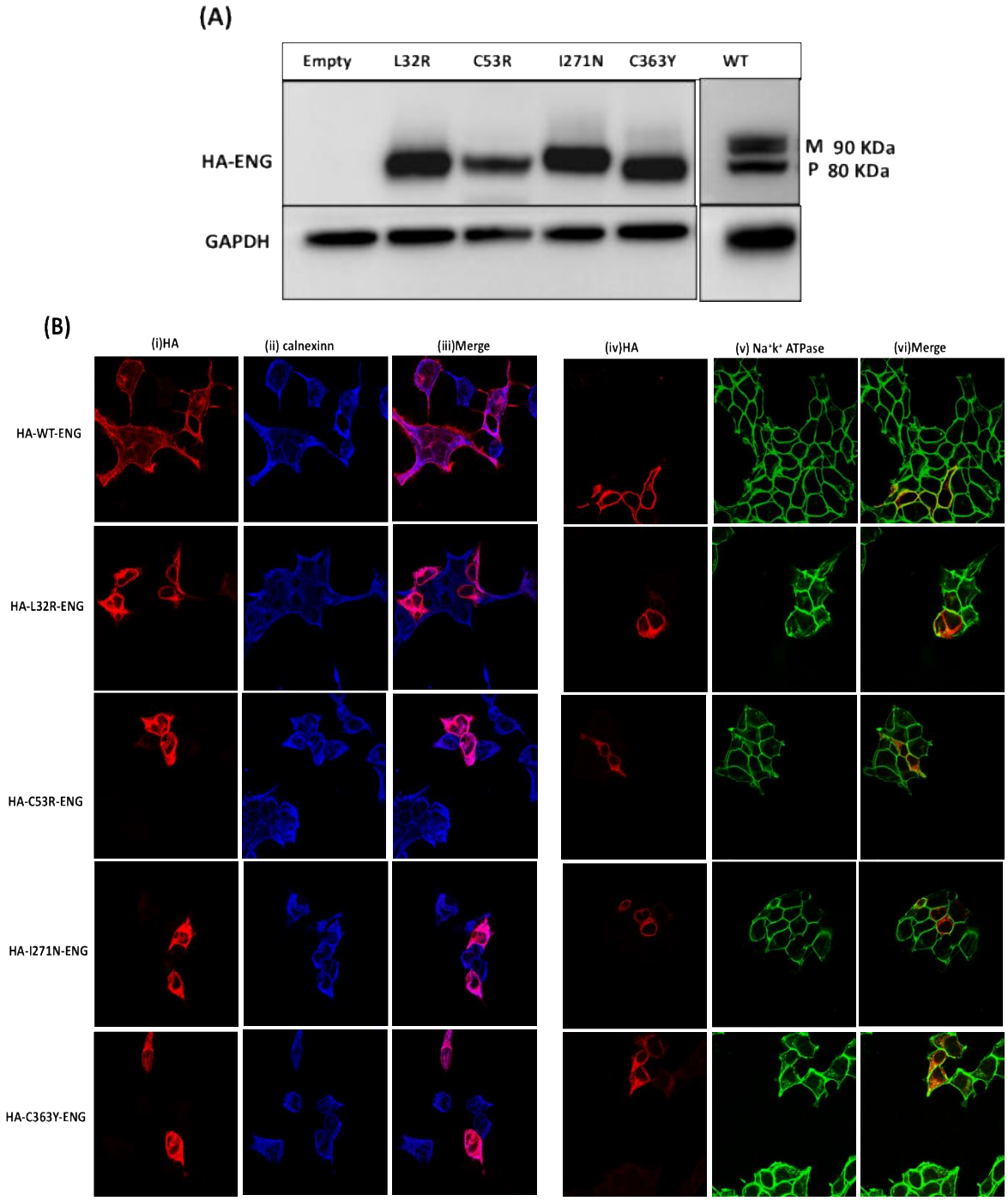

Figure 28:Validation of ER retention of transiently transfected HA tagged endoglin variants L32R, C53R, I271 and C363Y in HEK293T cells

(A)Total cell lysates were analyzed by immunoblotting against HA-tag. GAPDH was used as a loading control. (B)Subcellular localization of WT endoglin and mutant variants L32R, C53R, I271 and C363Y in HEK293T cells. Immunofluorescence images investigating co-localization of HA-tagged WT endoglin and mutant variants L32R, C53R, I271 and C363Y (Red, panels i and iv)) with ER marker (Calnexin, blue, panel (ii)) and plasma membrane marker Na^+K^+ATPase (green, panel v). A merged image of HA and Calnexin or Na^+K^+ATPase represented in (iii) and (vi), respectively. Images were taken using fluorescence confocal microscope at (100X). Abbreviations: (M) Mature endoglin, (P) Precursor endoglin

Aberrant trafficking behaviors of these mutants could be due to some physiochemical changes caused by the amino acid substitutions. Table 5 summarizes these changes, revealing that the introduced amino acids are generally larger in size, which could pose challenges for proper fitting and may distort the protein's 3D structure. Alterations in hydrophobicity can also impact hydrophobic interactions between residues located within the protein's core. Similarly, when a substitution results in a different charge, it can create a new electrochemical environment that may negatively impact protein-protein interactions. This is particularly true for variants L32R, C53R, and I271N, which are located in the orphan domain and play a role in facilitating protein-ligand interaction (Figure 29A, B & C). In addition, Residue C53 share a disulphide bridge with C182, therefore, substitution of the cysteine residue with Arginine is predicted to disrupt protein folding and interfere with the correct formation of the disulphide bridge at this position (Saito et al., 2017). Mutations in residues involved in intermolecular cysteine bridges situated in the Zona pellucida domain, such as C363Y can also have detrimental effects on the protein's tertiary structure, leading to consequences such as ER-retention and defective trafficking to the plasma membrane (Figure 29D) (Saito et al., 2017). In general, Zona pellucida domains known to mediated protein-protein interaction, however in endoglin it mainly facilitates homodimerization (Bokhove & Jovine, 2018; Saito et al., 2017). Table (5) also indicates the Grantham distance between the two amino acids (previously discussed in section 3.1) (Grantham, 1974). All Grantham distances are >100 and hence suggestive of radical substitution impact of function and structure of the protein.

All the mutation listed in Table 5 are predicted “pathogenic” according to the *insilico* prediction tool; Mutation Taster (MT) (https://www.mutationtaster.org/) and HOPE protein modelling software (https://www3.cmbi.umcn.nl/hope/). Moreover, in accordance with the established criteria set by The American College of Medical Genetics and Genomics (ACMG), these variants fulfill all the necessary requirements to be classified as "Pathogenic variants."(Richards et al., 2015). Nevertheless, variants L32R, I271N, and C363Y are currently labelled as "pending classification" in the University of Utah Department of Pathology HHT and *ENG* mutation Database

(http://www.arup.utah.edu/database/ENG/ENG_welcome.php, which suggests a delay in aligning with the latest scientific evidence and updated criteria outlined in guidelines.

Table 5: Physical and chemical properties of substituted residues in endoglin variants

Endoglin Variant	**L32R**	**C53R**	**I271N**	**C363Y**
WT residue	Leucine	Cysteine	Isoleucine	Cysteine
Mutant Residue	Arginine	Arginine	Asparagine	Tyrosine
Domain	Orphan	Orphan	Orphan	ZP-N
WT Charge	Neutral	Neutral	Neutral	Neutral
Mutant Charge	Positive	Positive	Polar	Polar
Mutant Size	Bigger than WT	Bigger than WT	Bigger than WT	Bigger than WT
Mutant Hydrophobicity	More hydrophobic than WT	Less hydrophobic than WT	Less hydrophobic than WT	Less hydrophobic than WT
Grantham Distances	102	180	149	194
***Mutant Pathogenicity**	Pathogenic	Pathogenic	Pathogenic	Pathogenic

*Pathogenicity of the variant was assessed using Mutation Taster (https://www.mutationtaster.org/). Underlined means “pending classification” in the *ENG* mutation database

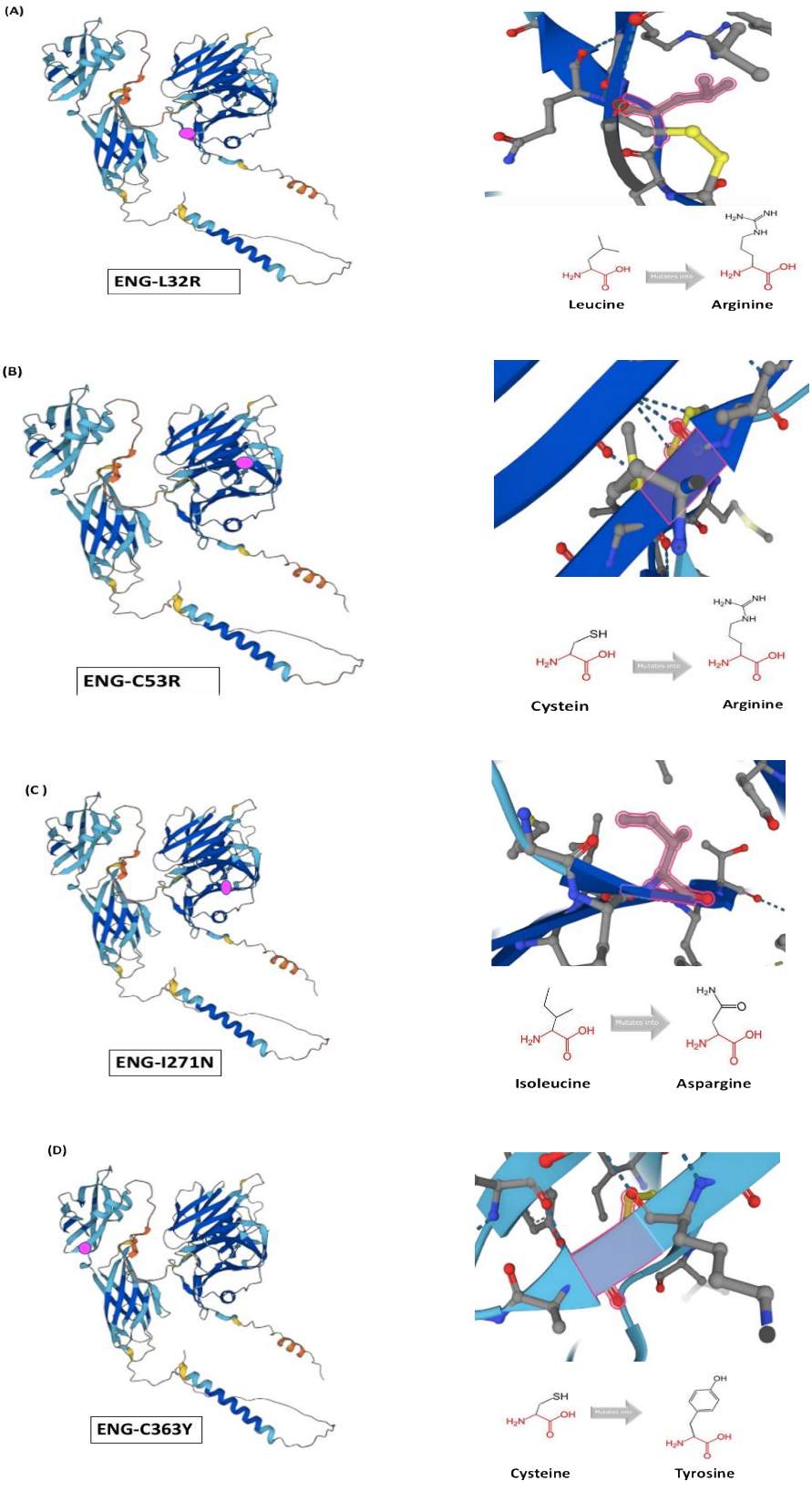

Figure 29:Predicted structure of endoglin variants

(A), (B), (C) and (D) shows 3D-structures of variant L32R, C53R, I271N and C363Y, respectively. It also shows the chemical structure of WT residue and its substitute. Zoomed in focus is on the location of the WT residue highlighted with red borders. Adapted from AlphaFold database.

To test the possible dominant negative effects exerted on WT endoglin by HA-tagged mutant variants L32R, C53R, I271N and C363Y, they were co-transfected with the pCMV6 vector harboring Myc-tagged WT endoglin. The empty vector was used as a negative control. Transfection was carried out at ratios of (1HA: 1Myc) and (4HA: 1Myc). Total cell lysates were probed with the relevant antibodies to confirm the expression of the proteins. In addition, equivalent protein amounts from these lysates were also immunoprecipitated with anti-HA agarose beads, followed by Western blotting probing with an anti-Myc antibody, as previously described. Western blotting results for HA-immunoprecipitates probed with anti Myc antibody are showing clear interaction between the immature band of Myc-WT and all four variants, similar to what previously observed for variant P165L and V105D (Figure 30B lanes 1, 2, 3, 4, 5). Densitometric analysis of the input blots is showing a significant reduction in the level of Myc-WT endoglin maturation when co-expressed with variants L32R, I271N and C363Y (Figure 30A lanes 1, 2, 3, 4, 5). The level of maturation in Myc-WT endoglin has dropped significantly from 60%, when transfected with an empty vector, to approximately 24%, 14%, and 39% when expressed in 1:1 ratio with L32R, I271N, and C363R, respectively, as depicted in the Figure 30C. Furthermore, co-transfection with an excessive amount of the mutants ER-retained variants at a DNA ratio of (4HA:1Myc) resulted in a further reduction in the maturation of Myc-WT, dropping from ~57% to 18%, 23%, and 28% with L32R, I271N, and C363R, respectively (Figure 30F). On the other hand, co-transfection with variant C53R have not affected the level of mature Myc-WT, which remained stable at approximately 51% and 46% when co-transfection was carried out at ratios 1HA:1Myc and 4HA:1Myc, respectively (Figure 30C & F). This unexpected finding may be explained by the potential instability of variant C53R, as evidenced by lower expression levels observed in the input blots compared to all other variants (Figure 30A & D), or reduced interaction with the WT protein.

To provide additional evidence that heterodimerization with ER- retained mutant variants is the sole reason for the significant depletion of mature Myc-tagged WT endoglin observed, we reversed the transfection ratio to expressing an excess of the

Myc-tagged WT endoglin, this time in a ratio of (1HA:4Myc) (Figure 30G). Our Western blotting results revealed that normal pattern of expression and trafficking of the mature Myc-WT was observed, when co-expressed with the mutant variants L32R, C53R, I271N and C363Y at this ratio. This was consistent with the pattern observed when co-transfected with HA-WT or the empty vector (Figure 30E, compare lane 1 with lanes 2, 3, 4 & 5). It is however also possible that the amount of the expressed mutant protein is too small to show detectable effect. Immunoprecipitation blots also confirm the heterodimerization of Myc-WT endoglin with the four tested ER-retained mutant variants L32R, C53R, I271N and C363Y, depicted by the presence of a single lower molecular weight band representing the immature precursor protein (Figure 30H lanes 1, 2, 3, 4 & 5).

Notably, analysis for inputs with Anti-HA antibodies to detect the L32R, C53R, I271N, and C363Y variants has also revealed the appearance of faint smears at 90KDa when co-expressed with Myc-WT endoglin at ratios 1HA:1Myc or 4HA:1Myc (Figure 30A & D lanes 1, 2, 3, 4 & 5). This observation may suggest that these mutant variants, which are typically trapped in the ER and appear as tight single lower molecular weight band, possibly demonstrate some maturation when co-expressed with WT endoglin. Is WT endoglin partially rescuing the mutant variants from ER retention or is this just an artefact? Notably, co-transfection of Myc-WT with variants P165L and V105D did not exhibit any level of maturation, making these results particularly intriguing. WT proteins have the potential to interact with and rescue mutants ER-trapped proteins through a process called complementation has been documented in the past. However, the success of this process depends on the type of mutation and the level of interaction between the proteins. Nevertheless, maturation of ER trapped variants could sometimes occur due to an excess expression of the mutant variants. This observation will probably need to be further investigated in the future since an excess expression of Myc-WT endoglin has not generated a similar effect as observed in (Figure 30G lanes 1, 2, 3, 4 & 5).

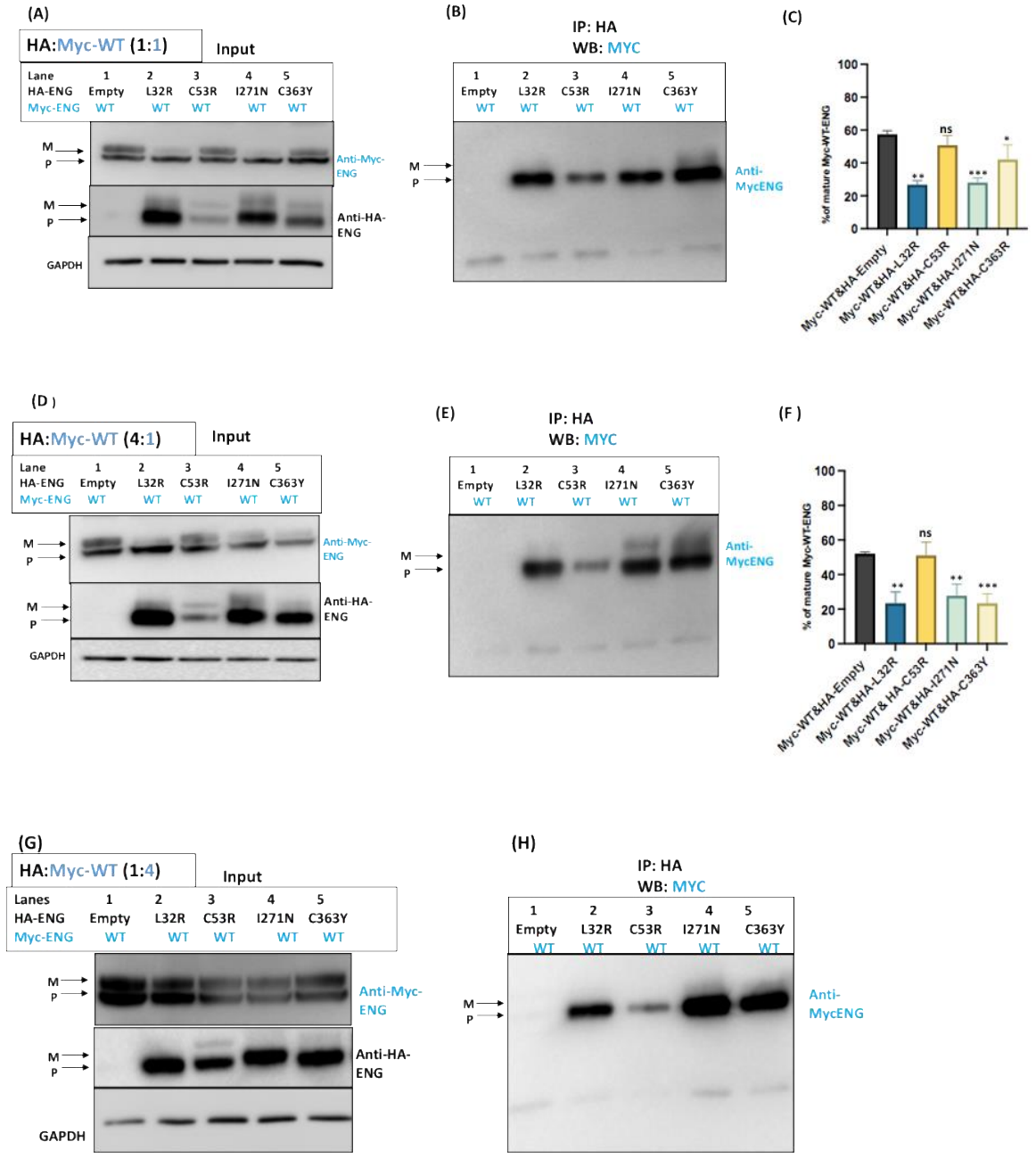

Figure 30: Formation of heterodimers between Myc-WT endoglin and variants L32R, I271N, and C363Y impairs the maturation of WT endoglin

(A) HEK293T cells were co transfected in a ratio of 1HA: 1Myc with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants L32R, C53R, I271N, and C363Y. Western blots of inputs were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-5). (B)Western blots showing HA immunoprecipitates probed with anti-Myc antibody (Lanes 1-5). (C) Bar graph representing mean densities of mature Myc-WT endoglin as a percentage of total protein expressed. Bars represent SEM from 3 experiments (n=3). Statistical significance of percentage of mature Myc-WT endoglin in (1HA: 1Myc) ratio with HA-tagged L32R, C53R, I271N, and C363Y relative its expression alone (empty vector) was determined using unpaired t-test. (*) $p \leq 0.05$; (**); $p \leq 0.01$; (***) $p \leq 0.001$. (D) HEK293 cells were co transfected in a ratio of (4HA:1Myc) with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants L32R, C53R, I271N, and C363Y. Western blots of inputs were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-5) (E) Western

blots showing HA immunoprecipitates probed with anti-Myc antibody (Lanes 1-5). (F) Bar graph representing mean densities of mature Myc-WT endoglin as a percentage of total protein expressed. Bars represent SEM from 3 experiments (n=3). Statistical significance of percentage of mature Myc-WT endoglin in (4 HA: 1Myc) ratio with HA- L32R, C53R, I271N, and C363Y relative its expression alone (empty vector) was determined using Unpaired t-test (GraphPad Prism) (*) $p \leq 0.05$; (**); $p \leq 0.01$; (***) $p \leq 0.001$. (G) HEK293 cells were co transfected with Myc-WT endoglin and HA-tagged constructs harboring mutant variants L32R, C53R, I271N, and C363Y in a ratio of 1HA:4Myc. Western blots of inputs were probed with anti Myc, anti HA and anti GAPDH (Lanes 1-4). (H)Western blots showing HA tagged mutant variants immunoprecipitates probed with anti-Myc antibody (Lanes 1-5). Abbreviations: (M) Mature endoglin, (P) Precursor endoglin

In order to further demonstrate the effects of those mutants on the maturation of Myc-WT endoglin, we analyzed the subcellular localization of Myc-WT endoglin in the presence of excessive amounts of the ER-retained mutants L32R, C53R, I271N and C363Y. Similar to the previous experiment, HEK293T cells were co-transfected with pCMV6 vector harboring Myc-WT endoglin and pCMV5 vector carrying one of the HA-tagged variants (L32R, C53R, I271N, C363Y or WT endoglin). The transfection was carried out at a DNA ratio of (4HA: 1Myc). Cells were immunostained against HA-tag representing HA-WT endoglin and mutant variants L32R, C53R, I271N & C363Y (Figure 31 Panel (i), red), Myc tag representing Myc-WT endoglin (Panel (ii), green) and Calnexin, ER marker, (panel (iii), blue). A merged image of the three is depicted in panel (iv). Similar to our earlier Western blot analysis, our results show that Myc-WT endoglin shows plasma membrane expression when co-expressed with an empty vector. Contrariwise, co-expression of Myc-WT endoglin with the ER retained mutant variants L32R, I271N, C363Y in a ratio of (4HA: 1Myc) resulted in a clearly observed colocalization of Myc-WT-endoglin with the ER calnexin marker and HA tagged mutant variants (Figure 31). On the other hand, Myc-WT endoglin shows plasma membrane localization when co-expressed with HA-WT endoglin, demonstrated by colocalization of the two WT endoglin proteins. Myc-WT has shown less colocalization with the Calnexin marker, when co-transfected with variant C53R, which agrees with our Western blotting analysis that maturation of WT-Myc has not been significantly affected by mutant variant C53R (Figure 31A & C lane 3).

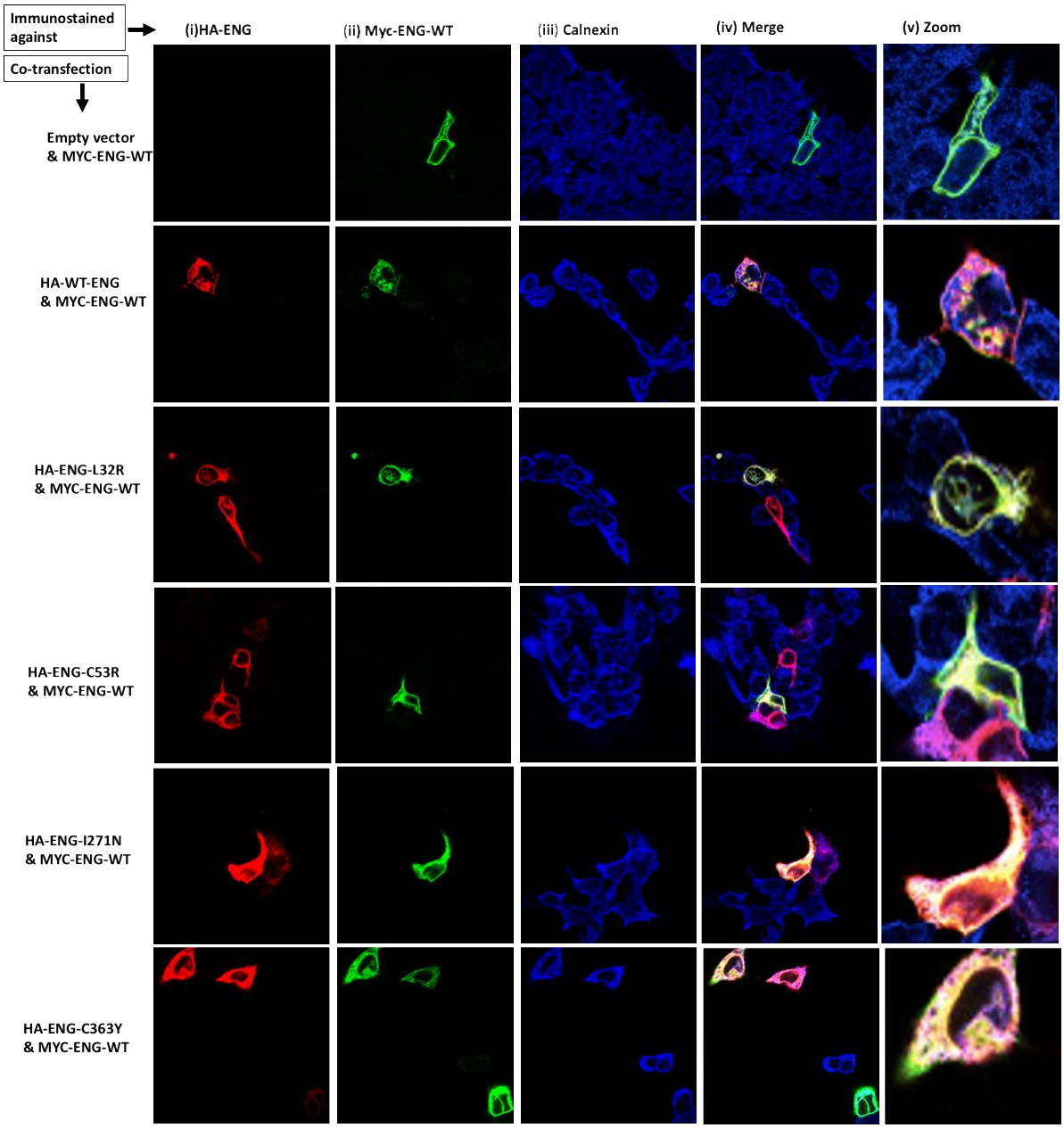

Figure 31: ER retention of Myc-WT endoglin under the dominant negative effect of ER mutant variants

HEK293T cells were co transfected in a ratio of 4HA: 1Myc with Myc-WT endoglin and HA-tagged constructs harboring WT endoglin and mutant variants L32R, C53R, I271N, and C363Y. Cells were immunostained against HA tag (Panel (i), red), Myc (Panel (ii), green) and Calnexin, ER marker, (panel (iii), blue). A merged image of the three is depicted in panel (iv). (v) is a zoomed in image of (iv).Images were taken using fluorescence confocal microscope at (100X).

3.7 Does the Dominant Negative Effect Exerted by ER- Retained Endoglin Mutant Variants Correlate with a More Severe Form of HHT1?

In this study we have demonstrated that ER- retained endoglin variants P165L, V105D, L32R, I271N, C363Y form heterodimers *in vivo* with WT endoglin. Formation of the heterodimers is predicted to negatively impact the trafficking of WT endoglin and may lead to its entrapment in the ER leading to additional loss of function. In autosomal dominant diseases such as HHT1, only one gene copy expresses the normal functional protein, therefore partial entrapment of this portion will further exacerbate the loss of function due to haploinsufficiency mechanism associated with HHT1. It is therefore predicted that variants exhibiting dominant negative effects are likely to be associated with a more severe disease phenotype. In theory, in these cases patients are expected to lose ~75% of the total gene function (50% due to the loss of function of the mutant allele and 25% due to dominant negative effects of the mutant allele on the WT allele).

In a preliminary and crude attempt to correlate the severity of the phenotype associated with variants P165L, V105D, L32R, I271N, C363Y, I have tried to carry out a simple phenotype/genotype correlation exercise. This exercise included 25 disease- causing endoglin variants that we have previously investigated at the subcellular localization level (Ali et al., 2011). Ten of these variants were found to be largely retained in the ER (including the variants studied in this thesis P165L, V105D, L32R, C53R, I271N, C363Y), the rest exhibited plasma membrane localization and therefore their cellular mechanism doesn't involve the ER quality control. After reviewing the studies reporting these variants, it was very difficult to assess the severity of each associated HHT1 case, as the clinical information presented in each study was very limited. Mostly, patients are recruited to the study if a clinical diagnosis of HHT1 is established according to Curacao criteria (Shovlin et al., 2000). Furthermore, information about the age of onset of HHT1 complications, which potentially could be used in scoring HHT1 severity was not clear enough to be able to establish a correlation assessment between age and disease phenotypes. Therefore, it was not possible to assess the severity of the phenotypes caused by variants exhibiting dominant negative traits. Our literature search has shown that patients carrying the variants P165L, V105D, L32R, C53R, I271N and C363Y are presented with classical HHT1 phenotype such as Epistaxis, telangiectases on multiple

sites, visceral telangiectases and family history (Table 6, highlighted in yellow). In order to investigate if there is a significant difference between the dominant negative mutants (6 variants) investigated in this thesis and the other variants (6 variants with available clinical data in Table 6), in terms “AVM” phenotype in HHT patients, I have conducted a chi-squared/ Fisher exact test analysis (SPSS software), and the results showed non-significant difference between the two groups, as shown in Table 7.

Identifying pathogenic variants associated with the severe form of the disease is crucial, despite the challenges posed by the heterogeneity of the HHT1 phenotype even among family members who carry the same variant. Dominant negative variants are prime candidates to be identified and closely monitored as they are predicted to be more damaging than other variants and cause more severe phenotype. Further detailed cellular mechanisms studies are needed especially for missense variants to establish if they would be retained in the ER and handled the ER associated protein degradation. In addition, closer collaborations between clinicians and cell biologists in this field should be encouraged.

Table 6: A summary of mutant variants of endoglin and the phenotype associated with affected individuals

Mutation Type	Age	Mutation at DNA Level	Mutation at Amino Acid Level	phenotype	Subcellular Localization	References
Missense	17y	c.494C>T	P165L	Epistaxis Telangiectases PAVM	ER	(Cymerman et al., 2003)
Missense	NA	c.314T>A	V105D	Epistaxis Telangiectases AVM FM	ER	(Bossler et al., 2006)
Missense	14 y	c479 T>	L32R	PAVM	ER	(Paquet et al., 2001)
Missense	Newborn	c.157T>C	C53R	PAVM CAVM FH	ER	(Pece-Barbara et al., 1999)
Missense	NA	c.812T>A	I271N	Epistaxis Telangiectases	ER	(Fontalba et al., 2008)
Missense	40y	c.1088G>A	C363Y	PAVM	ER	(Paquet et al., 2001)
Missense	NA	c.1146C>G	C382W	Confirmed diagnosis	ER	(Bayrak-Toydemir et al., 2004)
Missense	NA	c. 1144T>G	C382G	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Olivieri et al., 2007)
Missense	NA	c.145G>T	V49F	Confirmed diagnosis ‘‘Curacao criteria’’	ER	(Lesca et al., 2004)
Missense	NA	c.479C>A	A160D	PAVM	ER	(Yamaguchi et al., 1997)
Missense	NA	c.447G>C	W149C	Confirmed diagnosis	PM	(Gallione et al., 1998)
Missense	50y And 26y	c.740delT	A308D	Epistaxis PAVM CAVM FH	ER	(Bossler et al., 2006)
Missense	10 affected family members.	c.932T>G	V311G	Variable phenotype FH	PM	(Karabegovic et al., 2004)

Table 6: A summary of mutant variants of endoglin and the phenotype associated with affected individuals

Mutation Type	Age	Mutation at DNA Level	Mutation at Amino Acid Level	Phenotype	Subcellular Localization	References
Missense	NA	c.1204T>C	F403S	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Prigoda et al., 2006)
Missense	40y	c.1220 G>A	S407N	CAVM	PM	(Paquet et al., 2001)
Missense	NA	c.1234T>A	C412S	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Lesca et al., 2004)
Missense	NA	c.1238G>T	G413V	Telangiectases PAVM FM	PM	(Lux et al., 2000)
Missense	NA	c.1268A>G	N423S	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Song et al., 2016)
Missense	NA	c.1274C>G	A425G	PAVM	PM	(Fontalba et al., 2008)
Missense	NA	c.1309C>T	R437W	Epistaxis, telangiectasia	PM	(Bossler et al., 2006)
Missense	NA	c.1428G>C	Q476H	Epistaxis, telangiectasia	PM	(Wehner et al., 2006)
Missense	35y	c.1510G>A	V504M	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Lesca et al., 2004)
Missense	11y	c.1712G>A	R571H	Epistaxis, telangiectasia	PM	(McDonald et al., 2009)
Missense	53y	c.1844C>T	S615L	Confirmed diagnosis ‘‘Curacao criteria’’	PM	(Kuehl et al., 2005)

The Table summarizes information about 24 endoglin mutant variants reported in the Human Genetic Mutations Database HGMD (https://www.hgmd.cf.ac.uk/ac/search.php), and also previously investigated by our research

group (Ali et al., 2011). Clinical information of the patients was gathered from studies referenced in the Table.Abbreviations :Endoplasmic reticulum (ER), Plasma membrane (PM), Not Available (NA), Family History (FM), Pulmonary Arteriovenous Malformations (PAVM), Cerebral Arteriovenous Malformations (CAVMs).

Table 7: Chi-square and Fisher-Exact test statistical analysis

Group: Arteriovenous Malformation (AVM) Crosstabulation					
	Yes		**No**		**Total**
Dominant Negative Variants	5		1		6
Other Variants	3		3		6
Total	8		4		12
Chi square Test					
	Value	**DF**	**Asymptotic Significance**	**Exact Significance (2-Sided)**	**Exact Significance (1-Sided)**
Pearson Chi-Square	1.500	1	0.221		
Continuity Correction	0.375	1	0.540		
Likelihood Ratio	1.552	1	0.213		
Fisher's Exact Test				0.545	0.273
Linear-by-Linear Association	1.375	1	0.241		
No. of valid cases	12				

Chapter 4: Discussion

Components of the TGFβ receptor complex, including endoglin, are examples of plasma membrane proteins that enter the secretory pathway and get scrutinized by the ERQC system, transported to the Golgi for additional elaborate N- and O- glycosylation and then trafficked to their eventual functional subcellular location at the plasma membrane (Fisher et al., 2019). Some aberrant, but partially functional proteins can be prematurely selected for degradation by the ERQC mechanism, which can lead to their loss of function within the cells (Shao & Hegde, 2016). This has been shown to be the mechanism underlying numerous human genetic conditions (Chen et al., 2005; Guerriero & Brodsky 2012). For examples, our group has previously reported that defective trafficking can prevent membrane localization of endoglin, BMP type II receptor and ALK1 type 1 receptor, (Ali et al., 2011; Hume et al., 2013; John et al., 2015). This leads to defective folding, retention in the ER, followed by possible ERAD targeting and protein elimination.

In this study, we demonstrate that misfolded endoglin variants trapped in the ER are more stable than WT endoglin that is localized at the plasma membrane. Both P165L and V105D variants retained around 70% of their initial protein level until the end of the cycloheximide chase assay. On the other hand, endoglin WT showed significantly shorter half-life of 2 hours.The high stability of the two variants may be attributed to an extended folding process facilitated by components of the ERQC mechanism, followed by subsequent recognition and degradation through ERAD. However, a previous study conducted by Pece-Barbara et al. (1999) demonstrated that WT endoglin expressed in normal HUVEC samples reaches its peak level after approximately 2 hours and can persist for 4 extra hours. In contrast, our findings indicate a shorter half-life for WT endoglin (2 hours), which is likely due to the utilization of a different cellular model. HEK293 cells lack endogenous expression of endoglin, which implies that its turnover and processing in these cells could diverge from that in endoglin-expressing cells such as HUVEC (Pece-Barbara et al., 1999). Furthermore, it is worth noting that the application of cycloheximide as a translation inhibitor might have influenced regular cellular processes, potentially impacting the normal turnover of proteins. Nevertheless, our

findings unmistakably illustrate substantial and significant distinctions in the half-life and stability of wild-type endoglin compared to the P165L and V105D variants at all time points during the cycloheximide chase assay.

Our results have also shown that misfolded endoglin variants V105D and P165L are predominantly degraded through the proteasomal pathway, whereas endoglin WT is degraded through both the proteasomal and lysosomal pathways. These result were intriguing since the proteasomal system is essential for intracellular protein degradation, but an extracellular role of this degradation machinery has rarely been reported (Banik et al., 2020) (Sawada et al., 2002). However, this partial proteasomal degradation of the WT might also reflect that some of the WT might not actually mature and get retained in the ER and eventually gets exported and degraded by ERAD. As mentioned previously, protein folding process are error prone even for native protein without mutations, therefore WT membrane proteins are frequently degraded intracellularly through the proteasomal pathway when they temporarily fail to reach their proper conformation (Shao & Hegde, 2016). It is a process that has been adapted by the ERQC mechanism in order to maintain protein homeostasis in the ER. Nonetheless, our results also demonstrate that inhibition of the lysosomal pathway results in the accumulation of WT endoglin, which is most likely attributed to the fully glycosylated mature receptor protein localized at the plasma membrane. This finding could open a new therapeutic target that might relieve the haploinsufficiency state via moderate inhibition of WT endoglin lysosomal degradation. However, the process will require specific pharmacological agents which modulates WT endoglin degradation without an overall inhibition of the lysosomal pathway. Further studies are needed to delineate the detailed degradation of the plasma membrane endoglin. Lysosomal degradation has also been implicated in the degradation process of another TFGβ receptor; BMPR2, associated with familial Pulmonary hypertension (HPAH). It is a genetic disease characterized by elevated pulmonary pressure due defective arterial formation (Gomez-Puerto et al., 2019). The study demonstrates that in addition to proinflammatory cytokines, BMPR2 heterozygous mutation can cause augmentation of the autophagic influx, which could be secondary to the role of BMPR2 signaling in autophagy regulation (Gomez-Puerto et al., 2019). Our group has demonstrated in a previous study that a number of disease-causing variants of

BMPR2 are trapped in the ER (John et al., 2015). Therefore, defective trafficking followed by premature degradation of these variants through ERAD was proposed as the most likely mechanism underlying the disease's loss of function phenotype. Thus far, no study has investigated the degradation pathway of these variants and therefore the molecular mechanism underlying the disease pathology remains to be elucidated.

In this study, we have demonstrated that mutant variants trapped in the ER are prematurely degraded through the proteasomal pathway which directly implicates the ERAD mechanism in the degradation process. Variants P165L and V105D have shown a longer cellular half-life than the WT endoglin, possibly triggering the UPR mechanism and a cascade of events that usually leads to the elimination of misfolded proteins (Christianson & Ye, 2014; Wu & Rapoport, 2018). In order to further investigate the implication of ERAD components in the degradation pathway of these mutant variants, we utilized CRISPR Cas9 editing technology in order to investigate a direct involvement of major ERAD components in the degradation pathway of endoglin variants. Here, we have demonstrated that deficiency of HED1 E3 ubiquitin ligase in HEK293 cells causes accumulation of misfolded variants of endoglin retained in the ER. These results indicate that HRD1/SEL1L retro-translocon channel plays a key role in the elimination of these misfolded variants. In mammalians, a number of E3 ubiquitin ligases have been reported to play a role in the ubiquitination of ERAD substrates including HRD1, gp78, RMA1/RNF5, TEB4, TRC8/RNF139, RNF170, RNF103, and RFP2/TRIM1, which are all possible candidates for ERAD substrate ubiquitination (Christianson & Ye, 2014; Kadowaki et al., 2018). TRIM21/Ro52 E3 ubiquitin ligase was previously found to play a role in the proteasomal degradation of unfolded IgG1 (Takahata et al., 2008). Recently, it has also been shown to interact with soluble endoglin (sEng); a circulating proteolytic product of the transmembrane receptor protein (Gallardo-Vara et al., 2019). However, its role in the proteasomal degradation of mutant variants of endoglin transmembrane receptor remains to be elucidated. Nevertheless, the purpose behind the availability of numerous E3 ligases dedicated to ERAD substrates ubiquitination is still unclear. However, variability of ERAD substrates in terms of their target destination (cytosolic, plasma membrane, etc.) and mutation localization could be one of the reasons for the dedication of this huge number of E3 ubiquitin ligases embedded in the ER membrane.

HRD1 has been one of the most studied and best characterized E3 ubiquitin ligase and has recently been identified as a core component of ERAD substrates ubiquitination (Baldridge & Rapoport, 2016; Carvalho et al., 2010; Kadowaki et al., 2018; Schoebel et al., 2017; Taguchi et al., 2021; Wu et al., 2020). Loss of HRD1 has been reported to be directly linked to accumulation of amyloid β (Aβ) implicated in Alzheimer Disease (Kadowaki & Nishitoh, 2013). Accumulation of Aβ triggers the UPR mechanism which resolves the situation by inducing apoptosis, leading to neurodegeneration (Calabrò et al., 2021). HRD1 has also been shown to accelerate the degradation of cytotoxic aggregates of Polyglutamine (PolyQ) involved in development of Huntington Disease (HD) (Yang et al., 2007). Accumulation of toxic protein aggregated in HD, caused by an expansion of CAG repeats in the Huntingtin gene, is believed to contribute to neuronal dysfunction and cellular death (Yang et al., 2007). Interestingly, in our study, we see a reversed scenario where ERQC components such as HRD1 play a role in a premature degradation mechanism leading to a pathological condition characterized by loss of function traits. Lysosomal storage disorders such as Gaucher, Tay-Sachs and infantile GM1-gangliosidosis diseases caused by mutations that affects the native folding of lysosomal enzymes are also candidates for proteasomal degradation via the ERAD mechanism (Mohamed et al., 2020; Wang et al., 2011).

4.1 ERAD is a Potential Target for Therapeutic Interventions

Research studies have shown that a combination of ERAD inhibition and upregulation of folding cellular capacity can result in mutant enzyme rescue. ERAD inhibition was achieved through small molecules named kifunensine (Kif) and Eeyarestatin I (EerI), which inhibit ER mannosidase I and p97 ATPase activities, respectively (Wang et al., 2011). Inhibition of p97/VCP has also been used for the rescue of the mutant α1(A322D) subunit of the GABA inhibitory receptor associated with autosomal dominant juvenile myoclonic epilepsy. A combination of Eeyarestatin I and suberanilohydroxamic acid, a small molecule that enhances protein folding, restored surface expression of α1(A322D) subunits in cell lines (Han et al., 2015). Genetic diseases such as Wilson's disease and Progressive Familial Intrahepatic 1 cholestasis are also caused by mutations that affect the folding and the subsequent trafficking of the mutant proteins. Molecular chaperones and pharmacological means have been used to restore protein functionality (Hegde et al.,

2017). More recently, we have shown that the pharmacological chaperone N-n-butyl-deoxygalactonojirimycin enhances β-galactosidase processing and activity in fibroblasts of a patient with infantile GM1-gangliosidosis (Mohamed et al., 2020). Our research group have also proposed the modulation of mutant proteins containing the Frizzled Cysteine-Rich Domain (FZ-CRD) as a potential target for therapeutic interventions. (Milhem & Ali, 2019). Recently, ER stress has also been implicated in the pathogenic mechanism of Granular Corneal Dystrophy Type 2 (GCD2), caused by mutations in the TGFβ-Binding Protein B1(TGFB1), and characterized by the accumulation of abnormal protein deposits called granules in the cornea. The ER quality control mechanism delays the secretion of mutant TGFβ-Induced Protein (TGFβIp) through the ER/ Golgi secretory pathway (Choi et al., 2016). The use of 4-PBA as therapeutic agent for GCD2 caused significant reduction in the levels of TGFβIp, BiP, and ER stress kinases in GCD2 corneal fibroblasts. These results strongly suggest that the ER quality control system plays a key role in the pathogenesis of GCD2 and proposed the chemical chaperone 4-PBA as a target therapy for this disease (Choi et al., 2016). Melatonin, which is known to suppress cell death through reduction of the UPR or ER stress, has also been used *in vitro* to relief ER stress in GCD2 corneal fibroblasts, however the exact mechanism of its action has not yet been clarified (Choi et al., 2017). In a study by Sobolewski et. al., they demonstrated the potential for chemical chaperones such as thapsigargin, glycerol and Sodium 4-Bhenylbutyrate (4-PBA) to rescue cell surface expression of mutant BMPR2 expressed in Hela cells. Restored functionality of the mutant receptor has been shown through observation of enhanced activation of SMAD1/3 signaling pathway downstream of the receptor (Sobolewski et al., 2008). In another study by Frump et al., chemical chaperones Tauroursodeoxycholic acid (TUDCA) and 4-PBA have also proven successful in partially restoring cell surface expression of mutant BMPR2 in HPAH Patient-derived lymphocytes and in pulmonary Endothelial cells (PECs) from HPAH mouse model (Frump et al., 2013).

In this study, we have investigated the potential rescue effect of chemical chaperone, 4-PBA and glycerol, on ER- retained mutant variants P165L and V105D. Based on our findings, it can be concluded that the two chemical chaperones have not elicited pronounce effect on the folding and trafficking of the two mutant variants of endoglin.

However, it is very well acknowledged that the efficiency of chemical chaperones to elicit a rescue effect is dependent on the nature of the substrate protein as well as the extent of conformation distortion caused by that specific mutation. We have also observed that inhibition of the proteasomal pathway through the use of proteasomal and ERAD inhibitors (MG132, Kifunesnsine and of Eeyarestatin I, respectively), has resulted in significant accumulation of the mutant variants. However, accumulation of these mutants in the ER has not triggered the ERQC mechanism to facilitate a rescue effect. In fact, these mutants were stable in a precursor form for 24 hours before being degraded through the proteasomal pathway. Furthermore, a similar effect was observed when the mutant variants were expressed in HRD1 deficient cells. In conclusion, it is difficult to speculate on the underlying reasons for these results unless more work is carried out to investigate the cellular mechanisms that orchestrate the folding and trafficking of endoglin homodimer from the ER to the plasma membrane. On the other hand, genetic manipulation of the ER quality control mechanism has also been an approach adopted by many due to the latest advances in genetic editing tools. Recently, CRISPR-Cas9 technology has been utilized to knockout SEL1L adaptor protein in HEK293 cell line, in order to rescue Very Low-Density Lipoprotein Receptor (VLDLR) mutant protein responsible for Dysequilibrium syndrome (DES) (Ali et al., 2012). The degradation of pathogenic VLDLR through ERAD, which is primarily dependent on SEL1-L, was considerably delayed (Kizhakkedath et al., 2018; Kizhakkedath et al., 2019).

Premature mutant protein degradation through the proteasomal pathway has also been the underlying cause for certain classes of Cystic fibrosis (CF) (Meacham et al., 2001). Extensive work has been carried out in order to investigate CFTR biogenesis including protein folding and trafficking (Balch et al., 2011; Glozman et al., 2009). Identification of key players in the CF pathology has resulted in various classes of CF modulators, achieving remarkable progress in personalized treatment for CF patients (Lopes-Pacheco, 2016).

4.2 The Dominant Negative Effect: A Plausible Mechanism for Severe HHT1 Phenotype

The majority of the disease-causing mutations associated with HHT1 reported so far, lead to either unexpressed protein or proteins that are trapped in the ER (Ruiz-Llorente et al., 2017). Expression analysis of these mutants, together with the clinical phenotype associated with HHT1 in humans and heterozygous animal model supported the haploinsufficiency model as the main underlying mechanism for HHT1 (Ruiz-Llorente et al., 2017). Furthermore, HHT1 missense mutation mostly affect hydrophobic residues buried inside the tertiary conformation of the protein, which results in misfolded and functionally impaired protein. However, the haploinsufficiency model has not been able to explain the heterogeneity of the phenotypes amongst HHT patients. Many questions have been raised about the potential ability of some of endoglin variants to heterodimerize with WT endoglin and impair its functionality (Förg et al., 2014; Mallet et al., 2015). In this study we have identified five ER- retained variants that can heterodimerize with WT endoglin and adversely impact its trafficking to the plasma membrane in a dominant negative manner. Our findings indicate that variants P165L, V105D, L32R, I271N, and C363Y can potentially cause permanent retention of the WT endoglin protein, resulting in a compounded state of haploinsufficiency that is further exacerbated by a dominant negative effect. It has also been previously demonstrated that intracellular endoglin variants L170P and L262P also exhibited a dominant negative effect on WT type endoglin. Flow cytometry techniques have been used to measure the level of WT endoglin when co-transfected with mutant variants in HEK293 cell line (Mallet et al., 2015). On the other hand, earlier studies claimed that intracellular mutant variants are unable to form heterodimers with WT endoglin at the plasma membrane and hence are unlikely to exert a dominant negative effect (Pece et al., 1997). Their investigation was built around the hypothesis that mutant variants should be expressed at the plasma membrane in order to interfere with WT endoglin in a dominant negative manner. Around that time, the dominant negative effect model investigated variants that produce truncated soluble peptides that can bind and sequester binding ligands (McAllister et al., 1995). Although the dominant negative effect model was proposed nearly 30 years ago, it still has not been thoroughly investigated. Now, we have shown

that some ER-retained mutants can exert a dominant negative effect on the WT and impair its trafficking to the plasma membrane. These findings imply that mutant variants that exhibit normal trafficking to the plasma membrane can also form heterodimers with WT endoglin and potentially affect its normal function.

The dominant negative effect has been implicated in the pathology of various cellular processes. For example, mutant alpha synuclein protein, linked to Parkinson disease, exhibit dominant negative effects by disrupting the folding and stability of WT alpha-synuclein, leading to the formation of toxic aggregates in the brain (Benskey et al., 2016). Furthermore, the dominant negative effect can also be observed in cellular processes such as transcription and translation. Mutations in the p53 tumor suppressor protein can result in mutant forms that hinders the activity of the normal p53. The WT p53 is crucial in regulating cell cycle arrest and apoptosis, but its function is adversely affected by the dominant negative effect of the mutant p53. Consequently, the loss of p53 function increases the risk of cancer development (Willis et al., 2004). In a similar fashion, mutant proteins can disrupt translation by interfering with ribosomes or translation initiation factors. leukodystrophies are inherited disorders that are characterized by abnormalities in the white matter in the brain, that is responsible for neuronal transmissions (Li et al., 2004). These disorders have been linked to mutations in the translation initiation factor eIF2B (Li et al., 2004). Mutants were shown to exhibit a dominant negative effect on the WT protein resulting in abnormalities in protein synthesis leading to impairment of essential cellular functions (Keefe et al., 2020).

Chapter 5: Conclusions and Future Prospective

HHT1 is a rare genetic disease with a heterogeneous phenotype, ranging from few episodes of spontaneous nasal bleed to serious cerebral, pulmonary, hepatic or gastrointestinal AVMs. The symptoms can have a profound effect on the patients' quality of life and in severe cases, may pose a threat to their lives. Recently, several therapeutic interventions have emerged to manage a wide spectrum of symptoms depending on the site of lesions and the severity of the phenotype. However, currently there is no cure for HHT. In fact, the cellular and molecular mechanisms underlying the manifestation of the disease remain largely to be elucidated. In this thesis, I have shed a light on the molecular mechanisms associated with some ER-retained endoglin mutant variants. In a previous work, we have shown that some endoglin missense disease-causing mutant variants fail to be trafficked to the plasma membrane and get trapped in the ER, where they prematurely degrade. We predicted that these trapped mutant variants are most likely degraded through the ERAD mechanism, which targets misfolded proteins for ubiquitin proteasomal degradation. In this study, we have demonstrated that ERAD plays a key role in the proteasomal degradation of ER-retained endoglin mutant variants P165L and V105D. The implication of ERAD in the pathology of this disease opens a window for future therapeutic interventions that target the ERAD components. Nonetheless, the molecular mechanisms of endoglin biogenesis including chaperone assisted folding, trafficking and protein fidelity checkpoints must be further elucidated in order to design specific modulators for various types of endoglin variants. Despite the small number of research studies conducted for the purpose of restoring the functionality of disease-causing variants, the conceptual application of such therapeutic strategy has great potentials. In this thesis I have discussed many examples of the usage of genetic and pharmacological means in order to prolong protein retention in the ER, which lay the foundations for similar therapeutic strategies for HHT1. Cystic Fibrosis (CF) current interventional therapies represent a model example for future etiological therapies for HHT, and also other mutant secretory protein targeted by the ERQC mechanism for premature degradation. Similar to HHT1, ERAD has been implicated in the pathology of the disease. CF is commonly associated with a particular mutation in

the CFTR gene (F508del), expressing a misfolded protein that is selected by the ERAD mechanism for proteasomal degradation (Estabrooks & Brodsky, 2020). Therapies for CF have initially focused on managing the symptoms rather than targeting the cellular and molecular root of the disease. Then, extensive research work on the cellular mechanisms of the disease that regulates folding and trafficking CFTR channels, has led to the development of innovative therapies that modulate the defective variants (Buscarini et al., 2019). High throughput screening of drug-like compounds has led to the development of "corrector compounds" that enhance folding and trafficking of the mutant protein and also potentiator compounds that enhance their functional efficiency (Yang et al., 2003). The approval of the first potentiator drug (Ivacaftor) by the Food and Drug Administration (FDA) has pioneered a new era of mutation-specific drugs (Lander et al., 2001) . The drug has been designed specifically for patients carrying specific mutant variants such as G551D-CFTR, which involves a substitution of glycine (G) to aspartic acid (D) at position 551in the CFTR protein. In addition, other interventional therapies have focused on repairing the proteostasis environment instead of directly targeting the mutant variants. An example of this strategy is the use of histone deacetylase inhibitor such as Suberolyanilide Hydroxamic Acid (SAHA), that inhibits heat shock protein Hsp90 chaperone and facilitates maturation of CFTR mutant variant F508-del (Farinha & Canato, 2017). Extensive efforts in gene therapy has led to the development of F/HN-pseudotyped lentiviral vectors with high transfection efficiency in human Air–Liquid Interface (ALI) cultures (Alton et al., 2017).

Looking towards the future, the above examples address innovative therapeutic strategies which can be adopted in pursuit of effective HHT1 treatment options. However, many questions regarding the mechanisms that contribute to the disease pathology must first be resolved. In this study, we have demonstrated that some endoglin ER-retained mutant variants hijack the WT protein expressed by the functional allele, impeding its trafficking to the plasma membrane in a dominant negative manner. These findings imply that patients carrying these mutant variants are likely to lose up to 75% of the total expressed endoglin protein. In consequence, the dominant negative effect is predicted to further aggravate the haploinsufficiency state of the disease. Therefore, it is very important that these mutant variants are identified and investigated by geneticists and

molecular biologist. In addition, collaborative efforts with healthcare professionals will ensure that affected patients carrying variants associated with aggressive disease phenotype, are regularly monitored. The pilot study outlined in Table (6), which involves a preliminary genotype/phenotype correlation between the variants investigated in this thesis and other variants, presents a promising foundation for a collaborative effort between molecular geneticists and health professionals in regional or international specialized centers for HTT. This initiative holds potential for a large-scale collaborative project aimed at further advancing our understanding of HHT.A comprehensive library can be created, encompassing all reported variants along with their biogenesis, folding, functionality, and dominant negative traits. This information can then be correlated with well-defined phenotypic criteria established by specialized health professionals in the field. Undertaking such a project could aid in the identification of variants that exhibit potentially aggressive disease phenotypes. It is predicted, however, that family members carrying the same dominant negative variant may display variable disease phenotypes, further complicating investigations into the correlation between phenotype and genotype. Consequently, health professionals face the challenge of accurately categorizing the severity of the disease phenotype by considering all these variables.

Although this thesis focuses on HHT1-associated endoglin mutations, the general approach towards targeted interventional therapy may also be applied to HHT2 and HHT3 as well. Variability of the disease phenotype amongst HHT patients has made it difficult for clinicians to reach a conclusive clinical diagnosis at an early age unless a genetic test is carried out. In addition, the specificity of the organs affected in HHT phenotype has encouraged scientists to explore the possibility of a second hit phenomenon that may account for the development of the telangiectatic lesions at specific sites.

Although we have managed to shed a light on of the molecular mechanisms of some disease causing endoglin variants, we still need to extend our studies to a wider range of endoglin mutant variants. Furthermore, Plans have been made to validate some of our results in endothelial cells lines. However, the use of HEK293 cell line in this study has served the purpose of studying the pathology of mutant variants without an interference form an endogenous endoglin expression. Furthermore, the molecular mechanisms of

endoglin biogenesis including folding and trafficking must be elucidated in order to design specific modulators for various types of endoglin variants. We also call for collaborative cohort studies that can interpret genotype/phenotype correlation amongst HHT1 patients. The aim is to identify pathogenic variants that are potentially associated with severe disease phenotype with poor prognostic implications. This cohort must be kept under regular surveillance in order to ensure early identification of potentially life-threatening clinical presentations.

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List of Publications

Gariballa, N., P. Kizhakkedath, N. Akawi, A. John, and B. R. Ali. 2022. "endoglin Wild Type and Variants Associated With Hereditary Hemorrhagic Telangiectasia Type 1 Undergo Distinct Cellular Degradation Pathways." *Front Mol Biosci* 9:828199. doi: 10.3389/fmolb.2022.828199.

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Gariballa, N., A. Ben-Mahmoud, M. Komara, A. M. Al-Shamsi, A. John, B. R. Ali, and L. Al-Gazali. 2017. "A novel aberrant splice site mutation in COL27A1 is responsible for Steel syndrome and extension of the phenotype to include hearing loss." *Am J Med Genet A* 173 (5):1257-1263. doi: 10.1002/ajmg.a.38153.

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UAE UNIVERSITY DOCTORATE DISSERTATION NO. 2023:36

Brief description of the dissertation.

In this PhD thesis, molecular mechanisms implicated in the pathology of Hereditary Hemorrhagic Telangiectasia (HHT), that is caused by ER-retained endoglin mutant variants have been investigated. The implication of the ER-Associated Protein Degradation (ERAD) mechanism in HHT1 pathology has been demonstrated using various biochemical assays. Furthermore, we have shown that some ER-retained endoglin mutants bind and trap the wild type protein in the ER in a dominant negative manner.

Nesrin Mohammed Haider Gariballa received her PhD in Biomedical Sciences from the Department of Genetics & Genomics, College of Medicine & Health Sciences at UAE University, UAE. She received her Master of Science in Pharmacology and Biotechnology from Sheffield Hallam University, Sheffield, UK.

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