

KLHL24 mutation drives intermediate filament degradation, mitochondrial dysfunction and fibrosis in heart failure patients

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Aims

A striking aspect of epidermolysis bullosa patients with a mutation in *KLHL24* (*KLHL24^{mut}*) is their life-threatening deterioration of heart function. *KLHL24* is a component of the ubiquitin-proteasome system and acts as a substrate-specific adaptor protein for E3 ubiquitin ligase. *KLHL24^{mut}* is thought to represent a gain-of-function mutation, with associated cardiac and skin pathologies arising from the excessive degradation of its target proteins. Although reduced desmin levels in cardiomyocytes (CMs) have already been documented, the potential involvement of additional mechanisms in *KLHL24^{mut}*-driven heart pathology remains unexplored.

Methods and results

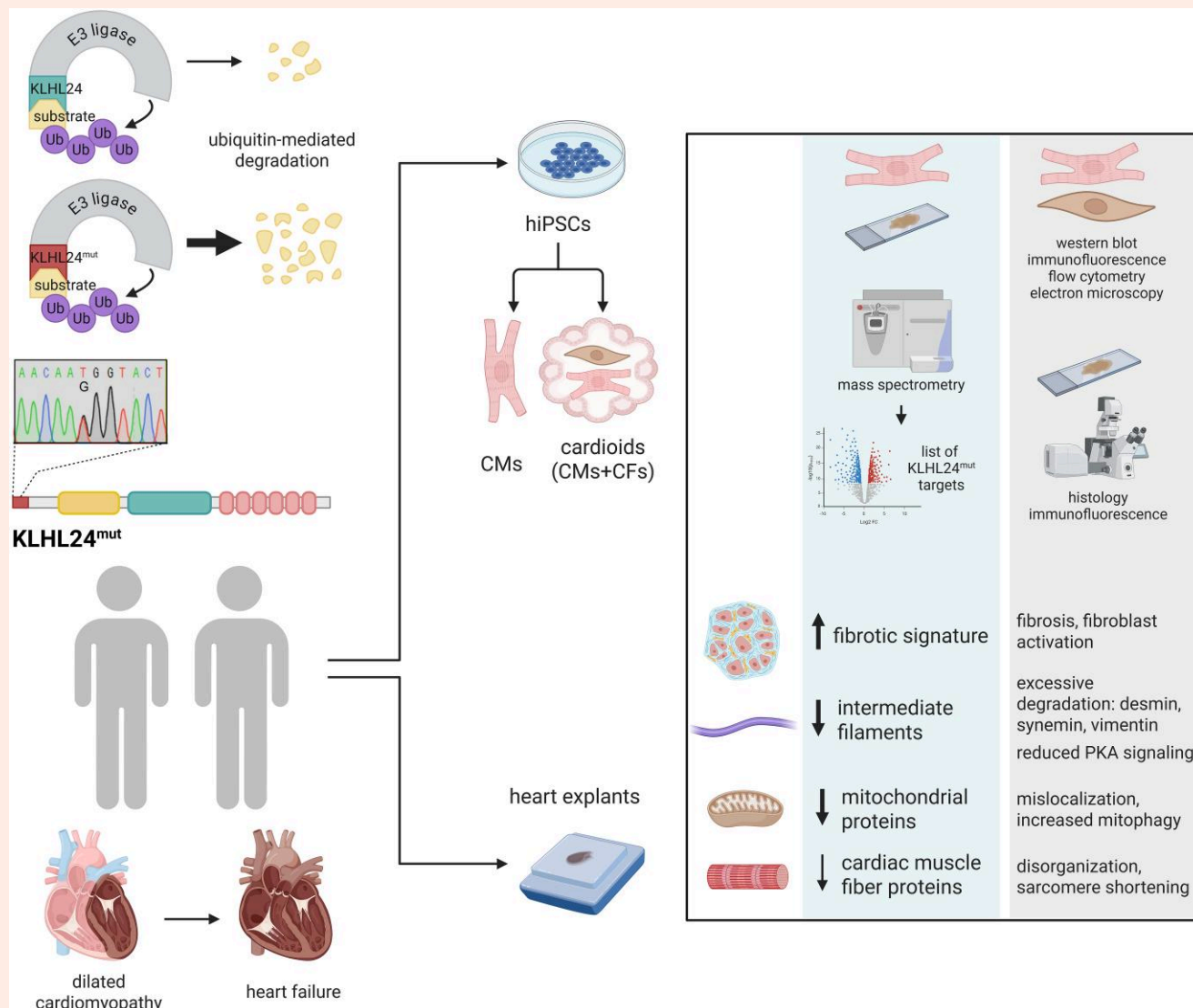
We report on two patients with *KLHL24^{mut}* who recently manifested heart failure. To gain insights into their physiopathology, we integrated clinical data with proteomic analyses of heart tissue as well as human induced pluripotent stem cell (hiPSC) models carrying *KLHL24^{mut}*. Mass spectrometry analysis of CMs differentiated from patient-derived hiPSCs mirrored the proteomic profile of their corresponding left ventricle tissue samples. *KLHL24^{mut}* resulted in a reduction of several intermediate filaments (IF), mitochondrial and muscle fibre proteins as well as the emergence of an early fibrotic signature. By utilising various hiPSC-derived cardiac models along with flow cytometry, immunofluorescence, and western blot analyses, we confirmed that the excessive proteasomal activity of endogenous *KLHL24^{mut}* caused a decrease in levels of desmin, synemin and vimentin, IF proteins of CMs and cardiac fibroblasts. Moreover, *KLHL24^{mut}* led to mitochondrial mislocalization and increased mitophagy, reduced PKA activity, and sarcomere shortening in CMs.

Conclusion

The deterioration of heart function in patients with *KLHL24^{mut}* is driven by excessive proteasome-dependent degradation of multiple IF proteins across various cardiac cell types. Monotypic hiPSC-derived CMs and end-stage patient-derived cardiac explants from patients exhibit similar features, uncovering early pathological mechanisms and identifying a list of potential novel *KLHL24^{mut}* target proteins. Finally, our findings validate that hiPSC-derived CMs represent a relevant model for future studies.

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Graphical abstract



Keywords

KLHL24 • Synemin • Desmin • Vimentin • Dilated cardiomyopathy

1. Introduction

Less than a decade has passed since it was discovered that mutations in the translation initiation codon of *KLHL24* (*KLHL24*^{mut}) cause a new form of epidermolysis bullosa simplex.^{1,2} Since then, it has become evident that *KLHL24*^{mut}-patients often exhibit pathologies beyond skin fragility, the most life-debilitating one being a high risk of developing dilated cardiomyopathy (DCM).^{3–5} The mechanisms causing these conditions are poorly understood. In the heart, *KLHL24* is expressed in all chambers and predominantly in cardiomyocytes (CMs), cardiac fibroblasts (CFs) and endothelial cells.⁶ *KLHL24* encoded protein, Kelch-like protein 24 (*KLHL24*), serves as a substrate-specific adaptor to Cullin 3-RING-E3 ubiquitin ligase, thus facilitating protein turnover via the ubiquitin-proteasome system. *KLHL24*^{mut} results in expression of a truncated protein lacking the first 28 amino acids (*KLHL24*^{mut}), which has been suggested to increase its stability.¹ Therefore, *KLHL24*^{mut}-mediated excessive degradation of targeted substrates likely underlies patient pathophysiology. All proteins implicated

as *KLHL24*^{mut} substrates thus far belong to the intermediate filament (IF) family. In skin, *KLHL24*^{mut} affects the turnover of epithelial keratins in keratinocytes^{1,7,8} and vimentin in dermal fibroblasts.⁹ In cardiac tissue, however, desmin is the only demonstrated downstream target of *KLHL24*^{mut} to date.¹⁰

To better understand the cardiac phenotype driven by *KLHL24*^{mut} and the underlying mechanisms, we combined clinical patient data with proteomic analysis. We present an overview of disease progression in several members of the previously described Chilean cohort from two families with *KLHL24*^{mut}, and the patient presentations of two related cohort members who recently manifested with heart failure.⁴ We investigate the role of *KLHL24*^{mut} using human induced pluripotent stem cells (hiPSCs) derived from these two patients, which we differentiate into cardiac models. We further validate our findings using proteomics of the patients' heart samples. We reveal that *KLHL24*^{mut} leads to the development of a cardiac fibrotic signature and drives defects in mitochondria, muscle fibres, PKA signalling activity and a reduction in several IF proteins. Our study

demonstrates that *KLHL24*^{mut} affects multiple cardiac cell types and has numerous downstream targets, most notably desmin and synemin in CMs and vimentin in CFs.

2. Methods

2.1 Clinical data and human tissue specimens

Clinical data on the Chilean cohort and anonymised CMR recordings were obtained with written informed consent. Samples of LV, RV, LA and RA from patients 1 and 2 were obtained after patient heart transplantation with written informed consent. Controls samples of LV and RV were obtained from the Pathology collection of Leiden University Medical Center and were sex- and age-matched to patients 1 and 2 (males, age 28–37). Control samples for LA and RA were obtained from postmortem anonymous donors (a female aged 37, two males aged 66), provided by the Pathology department of Leiden University Medical Center. Study conformed to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board at the Clínica Alemana de Santiago—Universidad del Desarrollo (#2013-145). The STROBE statement is included in the [Supplementary material online, Table S6](#).

2.2 Cell culture

045, 045 repaired, 046 and 046 repaired hiPSC lines were derived from fibroblasts from patients 1 and 2 and were previously described (named as LUMCi045, LUMCi045-A1, LUMCi046 and LUMCi046-A1, respectively).¹⁰ They were maintained in StemFlex™ medium (ThermoFisher Scientific, #A3349401) on vitronectin-coated plates (Stemcell Technologies, #07180) and passaged bi-weekly using Gentle Cell Dissociation Reagent (Stemcell Technologies, #07174). CMs and CFs were differentiated from 045, 045 repaired, 046 and 046 repaired following the published protocol.¹¹ CMs were passaged at day 19–21 with 2.5× TrypLE™ solution (ThermoFisher Scientific, #A121770) and maintained in Matrigel-coated plates (1:120, Corning® Matrigel® GFR, #354230) for up to day 40. They were cultured in LI-BPEL medium,¹¹ which was refreshed every 3 days. CFs were cultured in Fibroblast Growth Medium 3 (PromoCell, #C-23025) and refreshed every other day. Cardioids were generated as described before, with slight modifications to the protocol¹² (see [Supplementary Methods](#)). Brightfield images of cardioids were acquired using the EVOS XL Core Imaging System (ThermoFisher Scientific). All cultures were kept at 37°C, 5% CO₂ and 20% O₂.

2.3 Antibodies

Primary antibodies used are listed in [Supplementary material online, Table S1](#) and secondary antibodies are listed in [Supplementary Methods](#).

2.4 Immunofluorescence

Formaldehyde-fixed paraffin-embedded samples were sectioned, deparaffinized and heat-induced antigen retrieval was performed for 20 min, 98°C in EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9.0). Sections were blocked in 3% bovine serum albumin (BSA, Sigma Aldrich, #A8002-100G) for 1 h at room temperature (RT). Cardioids were embedded in Tissue-Tek OCT (Labtech, #16-004004). Cryosections were fixed in ice-cold acetone and blocked with 2% BSA PBS for 1 h at RT. hiPSC-derived CMs were plated on Matrigel (1:120, Corning® Matrigel® GFR, #354230)-coated coverslips or 96-well plates (Falcon, Black flat bottom, #353219) and CFs were plated on uncoated coverslips. CMs and CFs were fixed with 2% paraformaldehyde for 10 min, permeabilised with 0.2% Triton-X-100 for 5 min, and blocked with 2% BSA PBS for 1 h at RT. Paraffin sections, cryosections and fixed cells were incubated with primary antibodies in 2% BSA PBS for 60 min at RT (paraffin sections: overnight at 4°C), followed by incubation with secondary antibodies diluted 1:250 for 60 min at RT. Sections were mounted in Fluoromount-G® (SouthernBiotech, #0100-01) and coverslips in Mowiol. All samples were analysed with a Zeiss LSM900 Airyscan2 upright or LSM980 Airyscan NIR confocal microscope. Description of image analysis can be found in the [Supplementary Methods](#).

2.5 Flow cytometry

CMs were dissociated with 2.5× and cardioids with 5× TrypLE™. All samples were fixed and stained using the FIX&PERM™ Cell Permeabilization Kit (ThermoFisher, #GAS004). Stained single cell suspension in FACS buffer (1X PBS, 1% BSA, 0.5 mM EDTA) was analysed on a BD®LSR II Flow Cytometer, and the obtained data was processed using FlowJo-v10.10.0.

2.6 Western blot

Sub-confluent cell cultures were lysed in RIPA buffer supplemented with protease inhibitor (Roche, #11836170001) and phosphatase inhibitor cocktail 3 (Sigma-Aldrich, #P004). Lysates were cleared by centrifugation at 14,000 × g for 15 min at 4°C, diluted in Laemmli sample buffer, 100 mM DTT, and denatured at 98°C for 5 min. Proteins were separated by electrophoresis using 7.5% Criterion™TGX (BioRad, #64570068) or 4–12% Bolt™ Bis-Tris Plus (Invitrogen, #NWO4122BOX) gels, transferred to PVDF membrane, blocked for 1 h in 2% BSA TBST buffer and probed with antibodies. Bound antibodies were detected using Clarity™ Western ECL Substrate (Bio-Rad).

2.7 Mass spectrometry

For analysis of hiPSC-derived CMs, the purity of CMs was determined at day 40 using flow cytometry analysis of cardiac troponin T. 5 independent differentiation rounds of 045 (71–89% purity) and 4 rounds of 045 repaired (75–83% purity) were analysed. For mass spectrometry of LV tissue samples, 2 patient samples and 2 age-matched control LV samples were analysed. Detailed explanation of the sample preparation and analysis can be found in [Supplementary Methods](#).

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 10.2.2). An unpaired two-tailed t-test was used for comparisons of experimental groups with a control group. Multiple t-test with Holm-Sidak correction was used to analyse experiments with more than two groups. The significant values shown are described in the appropriate figure legends. Results with a *P* value lower than 0.05 were considered significantly different from the null hypothesis.

3. Results

3.1 Heart failure in two related patients carrying the *KLHL24* mutation

In 2019, a study was published on a cohort of seven Chilean patients from two families, carrying *KLHL24*^{mut}.⁴ Since then, progressive cardiac symptoms have been observed in four members of the cohort, most notably in two relatives who suffered heart failure in 2021 ([Table 1](#)) (patient numbers 14 and 15,⁴ from hereafter referred to as patients 1 and 2). Both patients were diagnosed with DCM with severely decreased left ventricular ejection fraction at a relatively young age ([Table 2](#)). A non-ischemic scar pattern was observed on late gadolinium enhancement (LGE) cardiac magnetic resonance (CMR) imaging, featuring extensive areas of subepicardial and mesocardial scar in basal and mid anterior/septal segments ([Figure 1A and B](#)). This pattern was consistent with those seen in non-ischemic DCM.¹³ Within four years after the initial presentation, both patients were hospitalised due to decompensated heart failure and ultimately received a heart transplant. The timeline of disease progression and detailed patient presentation for both patients can be found in [Table 2](#). Tissue samples from the left and right ventricle (LV and RV), as well as the left and right atrium (LA and RA), were collected from the heart explant of each patient. Histochemical analysis revealed prominent mixed and interstitial myocardial fibrosis with occasional fatty infiltration in the four heart chambers of both patients ([Figure 1C–E](#), [Supplementary material online, Figure S1](#)).

Table 1 A follow-up on the cardiac symptoms of the Chilean cohort of patients carrying the KLHL24 mutation

Patient number ⁴	Age in years (Sex)	Mutation KLHL24	Cardiac symptoms 2018 ⁴	Cardiac symptoms are currently present	ECG/Echocardiogram/CMR	Other relevant clinical information
12	33 (F)	c.[1A > G]	None	None	ECG (2023): normal results Echo (2023): normal results	Dyslipidemia
13	9 (M)	c.[2T > G]	None	None	ECG (2022): normal result Echo (2022): normal result	-
14	37 (M)	c.[2T > G]	Heart murmur, DCM	Underwent heart transplant (2021)	See Table 2	See Table 2
15	30 (M)	c.[2T > G]	Heart murmur, extrasystoles, dilated LV	Underwent heart transplant (2021)	See Table 2	See Table 2
16	38 (F)	c.[2T > G]	Heart murmur, mild systolic dysfunction of LV	Yes	CMR (2019): Mild systolic dysfunction and moderate LV dilatation with segmental wall motion abnormalities, predominantly in the lateral wall. Extensive late enhancement of the LV in a non-ischemic pattern consistent with genetic dilating cardiomyopathy. ECG (2023): Left axis deviation, normal atrial complexes. AV conduction is in the normal range. Intraventricular conduction compatible with incomplete right bundle branch block. Repolarisation with somewhat asymmetric negative T wave from V2 to V4 and biphasic in V5. Borderline QTc of 450 ms. Echo (2023): Mildly dilated LV with slightly depressed ejection fraction. Grade 1 diastolic dysfunction.	Hypercholesterolemia, hypertriglyceridemia
17	28 (F)	c.[2T > G]	None	Yes	CMR (2019): Global systolic function and volumes of both ventricles within normal limits. Extensive late enhancement with a non-ischemic pattern associated with oedema and the alterations described in mapping sequences, consistent with a genetic cardiomyopathy. ECG (2021): Normal electrical axis. Normal atrial and ventricular complexes. Normal AV and intraventricular conduction. Normal repolarisation. Single isolated ventricular premature beat. Borderline QTc of 450 ms. Echo (2022): Left ventricle of normal size, with walls of normal thickness and slightly decreased ejection fraction.	Hypercholesterolemia, hypertriglyceridemia
18	7 (M)	c.[2T > G]	none	No	ECG (2022): normal result Echo (2022): normal result	-

Abbreviations: AV: atrioventricular, CMR: cardiac magnetic resonance, DCM: dilated cardiomyopathy, ECG: electrocardiography, Echo: echocardiography, F: female, LV: left ventricle, M: male.

3.2 Proteomic analyses of hiPSC-derived CMs and patient left ventricle tissues

To gain insights into the physiopathology of KLHL24^{mut}-dependent DCM, we performed proteomic analyses on late-stage cardiac explants from patients with KLHL24^{mut} and on simplified *in vitro* CM monotypic cultures

from hiPSC lines harbouring KLHL24^{mut}. This approach aimed to identify potential novel target proteins of KLHL24. First, we generated CMs from patient-derived hiPSC lines (045 and 046 for patients 1 and 2, respectively) and their isogenic counterparts, where the KLHL24 mutation was repaired by gene editing (045 repaired and 046 repaired), as previously described¹⁴ (Figure 2A). Since KLHL24-mediated

Table 2 Timeline with detailed patient presentation for patients 1 and 2

YEAR	Patient 1	Patient 2
Initial physical examination	Length 1.68 m, weight 70 kg, BMI 24,8 kg/m ² , BSA 1.85m ²	Length 1.66 m, weight 70 kg, BMI 25,4 kg/m ² , BSA 1.85 m ²
2017	ECG: sinus rhythm 70 BPM, left axis deviation, normal AV-conduction and normal QRS morphology with a left anterior hemiblock. Repolarisation showed nonspecific alterations, with QTc time slightly prolonged (470 ms). HOLTER registrations: isolated ventricular extrasystoles without other arrhythmias. Echo: severe LV dilatation with diffuse hypokinesia and severely depressed systolic function (LVEF 26%). Moderate LA dilatation, functional mitral valve disease with mild regurgitation. No evidence of pulmonary hypertension.	ECG: sinus rhythm 71 BPM, normal heart axis, normal AV conduction and normal QRS morphology. Repolarisation showed nonspecific alterations, with QTc time slightly prolonged (480 ms). HOLTER registrations: isolated monomorphic ventricular extrasystoles without other arrhythmias. Echo: dilated LV with moderate systolic and diastolic function with normal filling pressures (LVEF 30%). Slightly dilated LA, normal dimensions of RA and RV. Functional moderate mitral regurgitation (Carpenter III). No evidence of pulmonary hypertension.
2019	LGE CMR: global hypokinetic contraction pattern of the dilated LV with akinesia of the basal and mid inferior and inferolateral segments (LVEF 25%, LVEDD 74 mm, LVEDV 207 mL; 115 mL/m ²). LGE shows nearly circumferential areas of subepicardial and mesocardial hyperintensity of the LV wall, most pronounced in the basal and mid anterior, anteroseptal and inferolateral segments, reflecting a non-ischemic pattern of fibrosis (see also Figure 1A). Pharmaceutical management is initiated with periodical follow-up.	LGE CMR: global hypokinesia of the LV myocardium with dyskinesia of the mid and apical inferior segments (LVEF 36%, LVEDD 76 mm, LVEDV 218 mL; 122 mL/m ²). LGE is present in widespread areas of the subepicardial and mesocardial LV wall, most pronounced in the basal, mid and apical septum and mid anterior segment, reflecting a non-ischemic pattern of fibrosis (see also Figure 1B). Pharmaceutical management is initiated with periodical follow-up.
2020	The patient presented with syncope due to fast monomorphic VT. Electrical cardioversion performed, ICD implantation	
2021	Hospitalisation due to decompensated heart failure. Echo: LVEF deterioration to 13% Heart transplant	Hospitalisation due to decompensated heart failure. Echo: deterioration, severe LV dilatation with eccentric hypertrophy, and severely compromised LVEF Heart transplant
Current status	Post-transplantation follow-up remains uncomplicated	Post-transplantation follow-up remains uncomplicated

Abbreviations: AV: atrioventricular, BPM: beats per minute, CMR: cardiac magnetic resonance, DCM: dilated cardiomyopathy, ECG: electrocardiography, Echo: echocardiography, ICD: implantable cardioverter-defibrillator, LA: left atrium, LGE: late gadolinium enhancement, LV: left ventricle, LVEF: left ventricular ejection fraction, RA: right atrium, RV: right ventricle.

cardiac pathologies typically manifest only after childhood⁴ (Table 1), we extended the CM culture period up to 40 days to promote CM maturation, as indicated by increased levels of *DES* and *KLHL24* mRNA,¹⁰ as well as an elevated *MYL2/MYL7* ratio compared to shorter culture periods (see Supplementary material online, Figure S2A–B). No differences in cell death or CM hypertrophy were observed between *KLHL24*^{mut} and *KLHL24*^{wt} CMs at this stage (see Supplementary material online, Figure S2C–D). For late-stage disease analysis, we compared formalin-fixed LV tissue sections of patients 1 and 2 with two age-matched control LV samples. Mass spectrometry analyses yielded 8419 and 2237 protein hits in monogenic CM cultures and tissue samples, respectively. Abundance ratios between 045 and 045 repaired CMs ($P < 0.05$, log₂ ratio = 0.07), and patient vs. control tissue (number of peptides > 1, abundance ratio variability < 80), could be obtained for 6397 and 690 proteins, respectively (Figure 2A, Supplementary material online, Figure S3A, Supplementary material online, Tables S2–S4). As anticipated, the majority of significantly differential hits were reduced in *KLHL24*^{mut} CM, *in vitro*, confirming that *KLHL24*^{mut} facilitates excessive proteasomal degradation of target proteins.^{1,10} At the late-stage disease, 108 proteins were found to be reduced, and 102 proteins were increased in patient compared to control LV samples with abundance ratios of patient to control less than 0.6 and greater than 1.6, respectively. The substantial number of proteins identified at high levels in patients at late stages of DCM compared to those observed *in vitro* likely reflected the complex biological changes associated with end-stage heart failure. Strikingly, proteomic analyses revealed significant similarities between monogenic *in vitro* CM cultures and late-stage disease samples, suggesting that hiPSC-derived CMs serve as a valuable model for studying this pathology.

3.3 KLHL24 mutation drives reduction of several IF proteins

All known substrates of *KLHL24* are IF proteins, including desmin in the heart, and keratins and vimentin in the skin.^{1,2,7–10} In the mass spectrometry dataset from hiPSC-derived CM, we detected 22 IF proteins, 6 of which showed reduced levels, while the remaining 16 were unaffected by *KLHL24*^{mut} (Figure 2B). The unaffected IFs mainly consisted of epithelial keratins, which are common contaminants of mass spectrometry analysis,¹⁵ and of Type V nuclear lamins, which play an essential role in the maintenance of the structural integrity and the regulation of transcription within the cell nucleus. A small reduction of nestin, an IF protein that is transiently expressed in CMs during embryonic development,¹⁶ was observed in *KLHL24*^{mut} CMs, along with a reduction of keratins 8 and 18. Interestingly, ectopic expression of keratins 8 and 18 in the heart has been previously reported and thought to act as a cardioprotective response to stress.¹⁷ The most significant reductions among IF proteins were observed in desmin, synemin and vimentin (Figure 2B). This pattern was also reflected in the mass spectrometry data set of *KLHL24* patients, which identified 10 IF proteins (Figure 2C). Excluding epithelial keratins, we observed reductions in desmin, vimentin and synemin, while the levels of nuclear lamins remained unaffected in *KLHL24* patients. The only IF protein that differed between the analyses was nestin, which was unaffected in patient tissue but reduced in the hiPSC-derived *KLHL24*^{mut} CMs (Figure 2B and C).

Until now, desmin was the only protein known to be affected by *KLHL24*^{mut} in the heart.^{5,10,18} Immunofluorescence and western blot

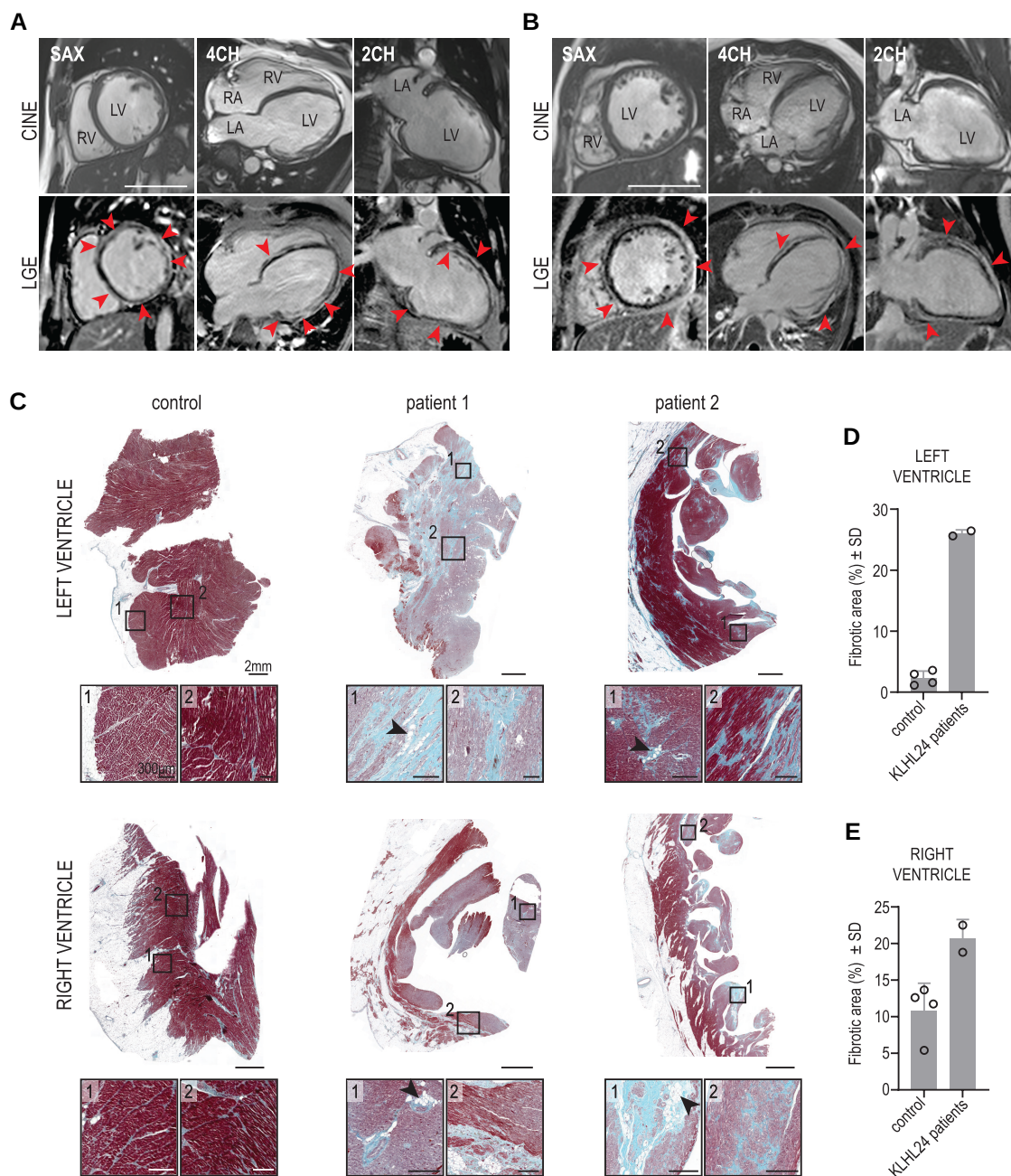
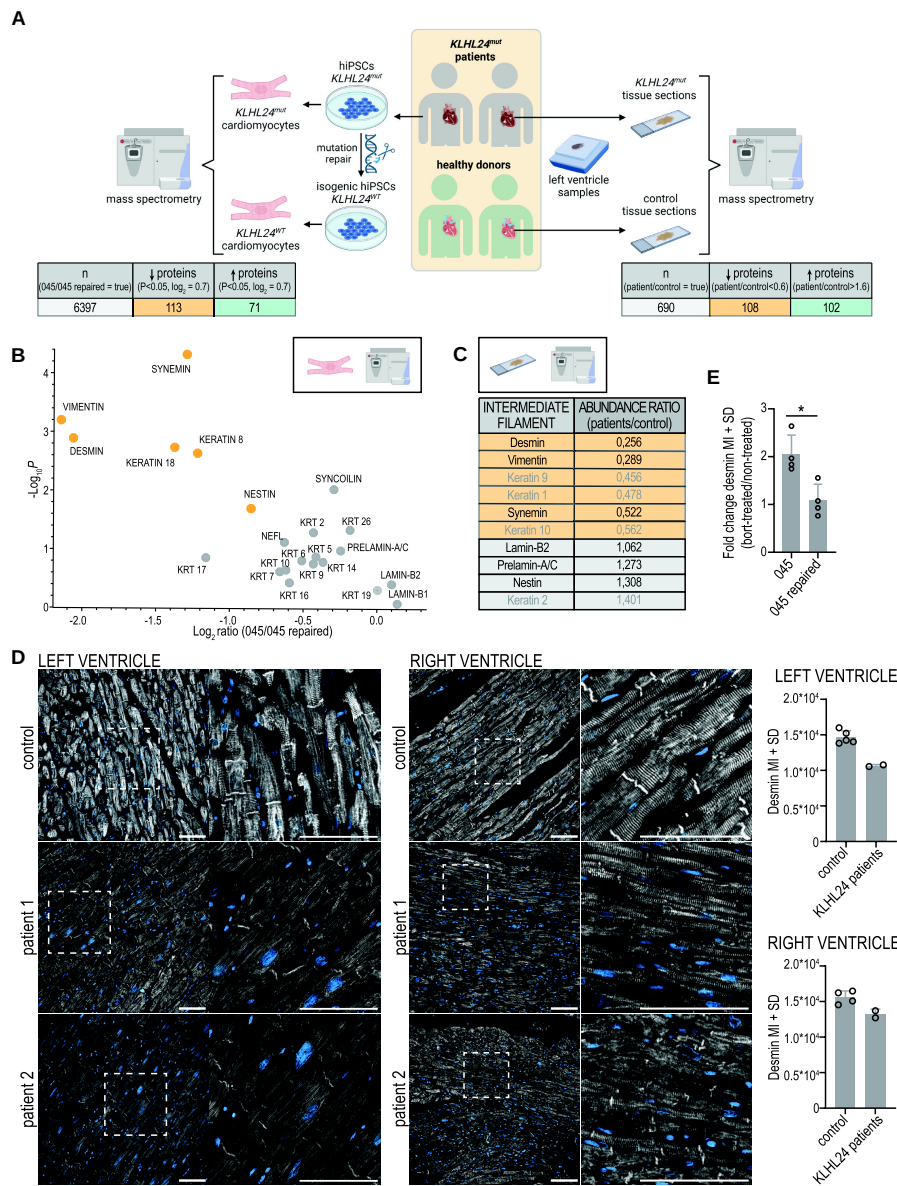


Figure 1 Heart failure in two related patients carrying the *KLHL24* mutation. (A–B) Cardiac magnetic resonance (CMR) images showing late gadolinium enhancement (LGE) of patients 1 (A) and 2 (B). CINE images are presented for reference. Scale bars: 50 mm. (A) LGE is observed in nearly circumferential areas subepicardially and mesocardially in the LV, most pronounced in the basal and mid anterior, antero-septal and inferolateral segments (red arrows) in patient 1 (B) and in widespread areas of the subepicardial and mesocardial LV wall, most pronounced in the basal, mid and apical septum and mid anterior segment (red arrows) in patient 2. (C) Masson-Goldner staining of the LV and RV of patients 1 and 2, with representative age-matched control tissue showing extensive mixed and interstitial myocardial fibrosis accompanied by fatty infiltrations (arrows) in patients. Squares: magnified areas. Scale bar: 2 mm (overview), 300µm (magnifications). (D–E) Quantification of Masson-Goldner stained fibrotic area in two *KLHL24* patients and age-matched control LV (D) and RV (E) samples. Each dot represents an independent donor ($n = 4$)/patient ($n = 2$) sample.

analysis confirmed that *KLHL24*^{mut} decreases desmin levels in CMs derived from both 045 and 046 hiPSCs, although its localisation remained unaffected (see [Supplementary material online, Figure S3B–C](#)). Consistently, desmin levels were reduced in the LV and RV of both *KLHL24*^{mut} patients compared to age-matched control tissues ([Figure 2D](#)). Interestingly, this reduction was not observed in the LA or RA of the same patients (see

[Supplementary material online, Figure S3D](#)). In line with *KLHL24*^{mut} driving desmin reduction through excessive proteasomal degradation,¹⁰ *DES* mRNA expression levels were similar in both patient-derived and repaired CMs (see [Supplementary material online, Figure S2A](#)). Treatment of CMs with a low dose of the proteasomal inhibitor bortezomib, which preserves their beating capacity, significantly increased desmin levels in 045 but not in



045-repaired CMs (Figure 2E, Supplementary material online, Figure S3E), demonstrating that proteasomal activity plays a significant role in endogenous KLHL24-mediated regulation of desmin.

3.4 Reduced synemin and PKA signalling activity in KLHL24^{mut} CMs

Mass spectrometry screen revealed two novel IF protein targets of KLHL24^{mut}: synemin and vimentin (Figure 2B and C). Synemin plays a structural and signalling role in CMs, where it forms heteropolymers with desmin or vimentin and interconnects Z-disks with desmosomes. Several cases of DCM patients with a mutation in synemin have been reported,¹⁹ suggesting that synemin may play a key role in KLHL24^{mut}-induced cardiac pathologies. Immunofluorescence analysis confirmed that, similar to desmin, KLHL24^{mut} significantly reduced synemin levels in both 045 and 046 CMs, without affecting its localisation to Z-disks (Figure 3A). Additionally, both major synemin isoforms, α - and β -synemin, were significantly reduced in 045 and 046 CMs compared to their isogenic controls (Figure 3B, Supplementary material online, Figure S4A). Synemin levels were clearly reduced in patient LV, RV, LA and RA samples compared to the age-matched controls, although its localisation to the sarcomeres, where it co-localizes with plectin, remained unaffected (Figure 3C and D, Supplementary material online, Figure S5). Since KLHL24^{mut} did not affect synemin mRNA expression levels (Figure 3E), we hypothesised that, like desmin, synemin is regulated by proteasomal degradation. Indeed, bortezomib treatment significantly increased both synemin isoforms in 046 CMs, but not in 046-repaired CMs (Figure 3F, Supplementary material online, Figure S4B). Given the interconnection between desmin and synemin, we investigated whether their regulation might be co-dependent in KLHL24^{mut} CMs. The markedly elevated desmin levels observed in the atria of KLHL24 patients—where synemin is significantly reduced—suggest that desmin is unlikely to act upstream of synemin (see Supplementary material online, Figure S3D, S5). Moreover, overexpression of synemin in KLHL24^{mut} CMs did not lead to increased desmin levels (see Supplementary material online, Figure S4C), indicating that the proteasomal degradation of desmin and synemin is regulated independently.

Synemin also functions as a Protein kinase A (PKA)-anchoring protein, modulating key cardiac signalling pathways.²⁰ To determine whether its reduction affects PKA signalling in KLHL24^{mut} CMs, we assessed the phosphorylation of CREB, a downstream PKA target. KLHL24^{mut} CMs showed significantly decreased pCREB levels compared to isogenic control (Figure 3G, Supplementary material online, Figure S4D), demonstrating that KLHL24 mutation leads to impaired PKA signalling.

3.5 Vimentin is a novel substrate of KLHL24 in the heart

Although vimentin predominantly localises to mesenchymal cells in adult tissues, it is expressed by CMs during fetal development.²¹ Its presence in the mass spectrometry analysis could thus originate from both immature hiPSC-derived CMs and mesenchymal cells, which are commonly present during CM differentiation. A recent study demonstrated that KLHL24^{mut} promotes vimentin degradation in dermal fibroblasts, impairing wound healing and dermal fibroblast activation.⁹ This led us to investigate whether CFs are also affected by KLHL24^{mut}, potentially contributing to cardiac pathology in KLHL24 patients. No KLHL24^{mut}-mediated reduction in vimentin was observed in monolayer cell cultures of hiPSC-derived CFs (see Supplementary material online, Figure S6A). However, since vimentin expression is often upregulated in cells cultured as a monolayer,²² we differentiated hiPSCs into cardioids, a more physiologically relevant organoid model. Cardioids co-differentiate CMs, CFs and endothelial cells to form a structure resembling LV¹² (see Supplementary material online, Figure S6B). Immunofluorescent analysis showed that, consistent with CM monolayers, KLHL24^{mut} CMs in cardioids exhibited reduced desmin levels (see Supplementary material online, Figure S6C). Dissociation of the cardioids followed by flow cytometry revealed that vimentin was expressed by both CFs and CMs, albeit at lower levels in CMs. Vimentin levels were significantly reduced in 045 and 046 CFs and CMs compared to their isogenic controls (Figure 4A–C). Bortezomib treatment

confirmed that, like desmin and synemin, vimentin reduction in KLHL24^{mut} CMs and CFs was due to excessive proteasomal degradation (Figure 4D, Supplementary material online, Figure S6D). Finally, analysis of cardiac tissue from patients 1 and 2 showed a clear reduction in vimentin levels across all four heart chambers (Figure 4E, Supplementary material online, Figure S5).

3.6 KLHL24 mutation leads to reduced mitochondrial and muscle function

Enrichment ontology clusters and interactome network analyses were performed on proteins with significantly altered abundance between pathological and control conditions. In addition to proteins associated with IF cytoskeleton organisation, the KLHL24^{mut} led to a reduction in proteins linked to mitochondrial function and metabolism and muscle function and/or development in both hiPSC-derived CMs and patient LVs (Figure 5A). Consistently, interactome network analysis of proteins reduced in KLHL24^{mut} CMs and KLHL24 patients revealed two distinct clusters: a mitochondrial cluster and a muscle cluster (Figure 5B and C).

The muscle interactome cluster in the CM dataset included proteins from the troponin complex, which are components of the cardiac muscle thin filaments (TNNI3, TNNT2, TNNC1); tropomyosins, which are sarcomeric components that associate with the troponin complex and regulate the interaction between myosin and actin (TPM1 and TPM2); myosin light chain proteins (MYL2, MYL3, MYL4, MYL9); the actin-binding protein leiomodin 2 (LMOD2), associated with infantile DCM²³; the calcium-handling protein calsequestrin (CASQ2); the sarcomeric protein Synaptopodin-2 (SYNPO2); the cardiac LIM protein CSRP3 and the sodium channel SCN5A, impaired function of which is a known cause of DCM,^{24,25} as well as IFs desmin and synemin. A smaller cluster in the network identified proteins involved in cell cycle progression and cell division (CDK1, KSB1, CDCA3, CDCA3) (Figure 5B). The KLHL24 patient data formed a smaller muscle interactome cluster, including myosin heavy chain (MYH3), telethonin (TCAP), a titin-associated Z-disc protein involved in DCM,²⁶ troponin C (TNNC1) and IF proteins vimentin, desmin and synemin (Figure 5C). Immunofluorescence analysis of hiPSC-derived CMs revealed reduced levels of the most differentially abundant muscle cluster protein, cardiac troponin I, in 045 and 046 CMs compared to their isogenic controls, with no obvious difference in localisation (see Supplementary material online, Figure S7A–B). mRNA expression analysis of TNNI3 and TNNT2 showed no differences between KLHL24^{mut} and control CMs (see Supplementary material online, Figure S7C), indicating that the downregulation of troponin complex proteins in CMs occurs at the post-translational level. Sarcomere structure analysis showed significantly reduced sarcomere length and organisation in 045 and 046 CMs compared to their isogenic controls (Figure 5D, Supplementary material online, Figure S7E). Interestingly, these changes did not result in functional differences in CM contractility at this disease stage, as KLHL24^{mut} and control CMs showed similar contraction amplitudes regardless of pacing frequency (see Supplementary material online, Figure S7D). This aligns with previous findings demonstrating that a high degree of CM maturity and model complexity, combined with prolonged exposure to a high level of dynamic load, are required to detect KLHL24^{mut}-induced CM contractility defects.¹⁰ This may explain why cardiac defects in patients are typically reported only in adulthood (Table 1). Furthermore, aberrant muscle function in KLHL24 patients was reflected in shorter sarcomeres in patient LVs as compared to the controls, although no differences in sarcomere organisation were observed between the two (Figure 5E, Supplementary material online, Figure S7F).

The mitochondrial interactome cluster in CM dataset contained several key mitochondrial proteins including subunits of mitochondrial respiratory complex I (NDUFA12, NDUFA13, NDUFB1, NDUFB3, NDUFB4, NDUFB5, NDUFV3, NDUFS4, NDUFS5, MT-ND1), complex IV (COX5B, COX6C, COX7C, COX7A2, COX4I1), mitochondrial ATP synthase (MT-ATP6 and ATP5PF), and two mitochondrial pyruvate carrier proteins (MPC1 and MPC2). Additionally, outside the main clusters but with a known link to cardiac disease via mitochondrial regulation function, heat shock protein HSPB2²⁷ was identified (Figure 5B). The mitochondrial cluster was also the most prominent in the KLHL24 patient dataset, containing numerous mitochondrial proteins, including those from respiratory

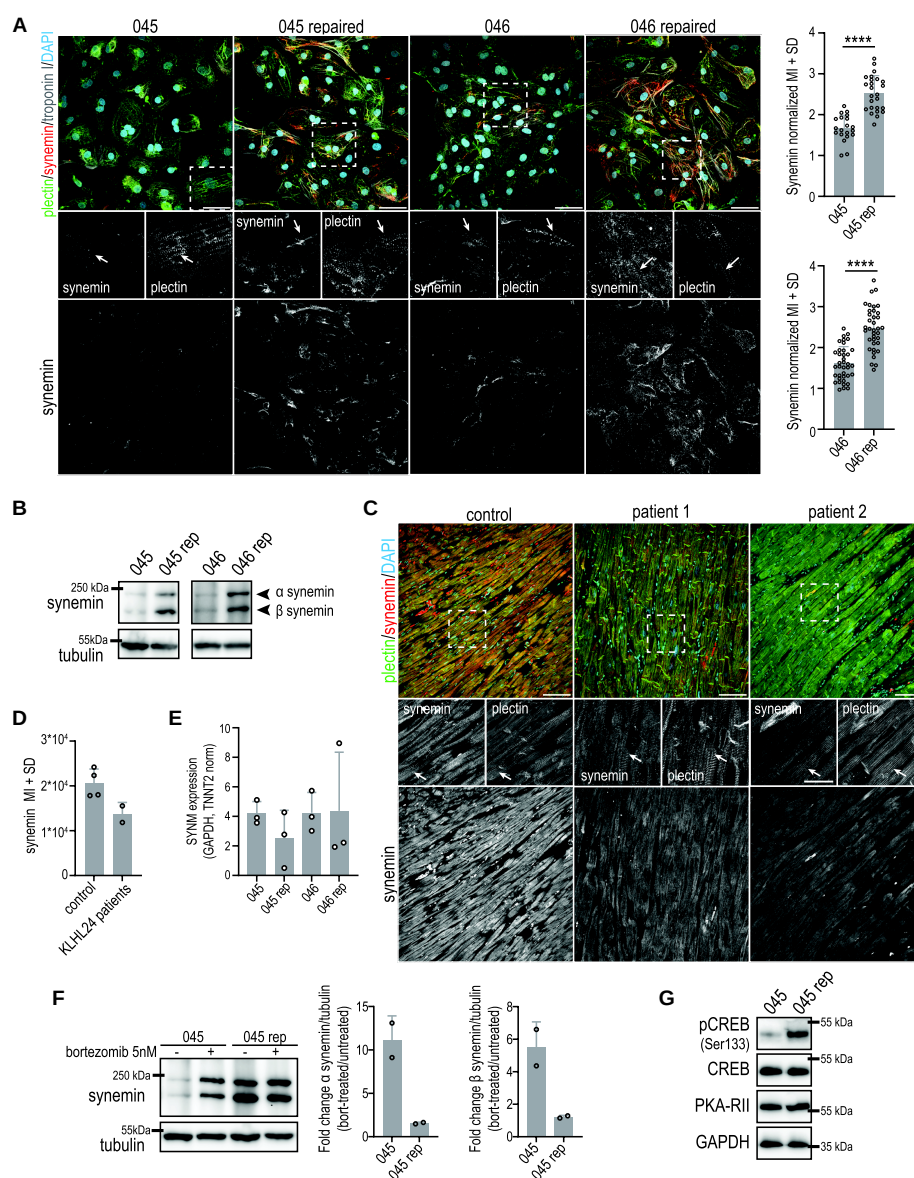


Figure 3 *KLHL24* mutation results in increased proteasomal degradation of synemin in CMs. (A) Representative confocal image of immunofluorescence staining (left) and quantifications (right) showing significantly reduced synemin levels in 045 and 046 as compared to 045-repaired and 046-repaired CMs. CMs were counter-stained for nuclei (DAPI) and plectin, which is located at Z-disks in CM sarcomeres, where it co-localises with synemin in *KLHL24*mut and control CMs (arrows). Confocal images are presented as maximum projected z-stacks. Dashed squares: magnified areas. Scale bar: 50 μ m (overview), 30 μ m (magnifications). Each dot represents a quantified image ($n = 20$ (045), 25 (045 repaired), 38 (046), 37 (046 repaired)). 2–4 independent differentiations of hiPSC were used for quantifications (normalised mean intensity $\pm$ SD, unpaired t -test, **** $P < 0.0001$). (B) Representative western blot of synemin and loading control (tubulin). Uncropped western blot and quantifications can be found in [Supplementary material online, Figure S4A](#). Both predominant isoforms of synemin are reduced in 045 and 046 CM lysates as compared to their isogenic controls. (C) Representative confocal images showing reduced synemin levels in LV tissue sections of patients as compared to their age-matched controls. Sections were counterstained for plectin. Synemin colocalises at z-lines with plectin in control as well as patient CMs (arrows). Dashed squares: magnified areas. Scale bar: 100 μ m (overview), 30 μ m (magnifications). (D) Quantification of panel C. Each dot represents an independent tissue sample ($n = 4$ (controls), 2 (patients)) and is an average of 10–11 images (mean $\pm$ SD). (E) Quantitative RT-PCR analysis of CMs differentiated from hiPSC and cultured for 30 days. Normalised ($\Delta\Delta C_q$) mRNA expression of synemin in 045, 045 repaired, 046 and 046 repaired CMs. mRNA expression of *GAPDH* and *TNNT2* was used for normalisation. Each dot represents an independent CM differentiation round and is an average of technical duplicates ($n = 3$, mean $\pm$ SD is shown). (F) Representative western blot (left) and quantifications (right) of synemin levels in 045 and 045 repaired CM lysates, untreated and treated with 5 nM bortezomib for 43 h. Bortezomib treatment results in strongly increased levels of α - and β -synemin isoforms in 045 but not in 045 repaired CMs. Each dot represents a lysate of independent CM differentiation and treatment ($n = 2$). Uncropped western blot and quantifications can be found in [Supplementary material online, Figure S4B](#). (G) Representative western blot of phosphorylated CREB (pCREB) and total CREB, a downstream target of PKA, as well as the regulatory subunit of PKA (PKA-RII), which interacts with synemin, and the loading control (GAPDH) in *KLHL24*^{mut} CMs. Uncropped western blots and quantifications can be found in the [Supplementary material online, Figure S4D](#).

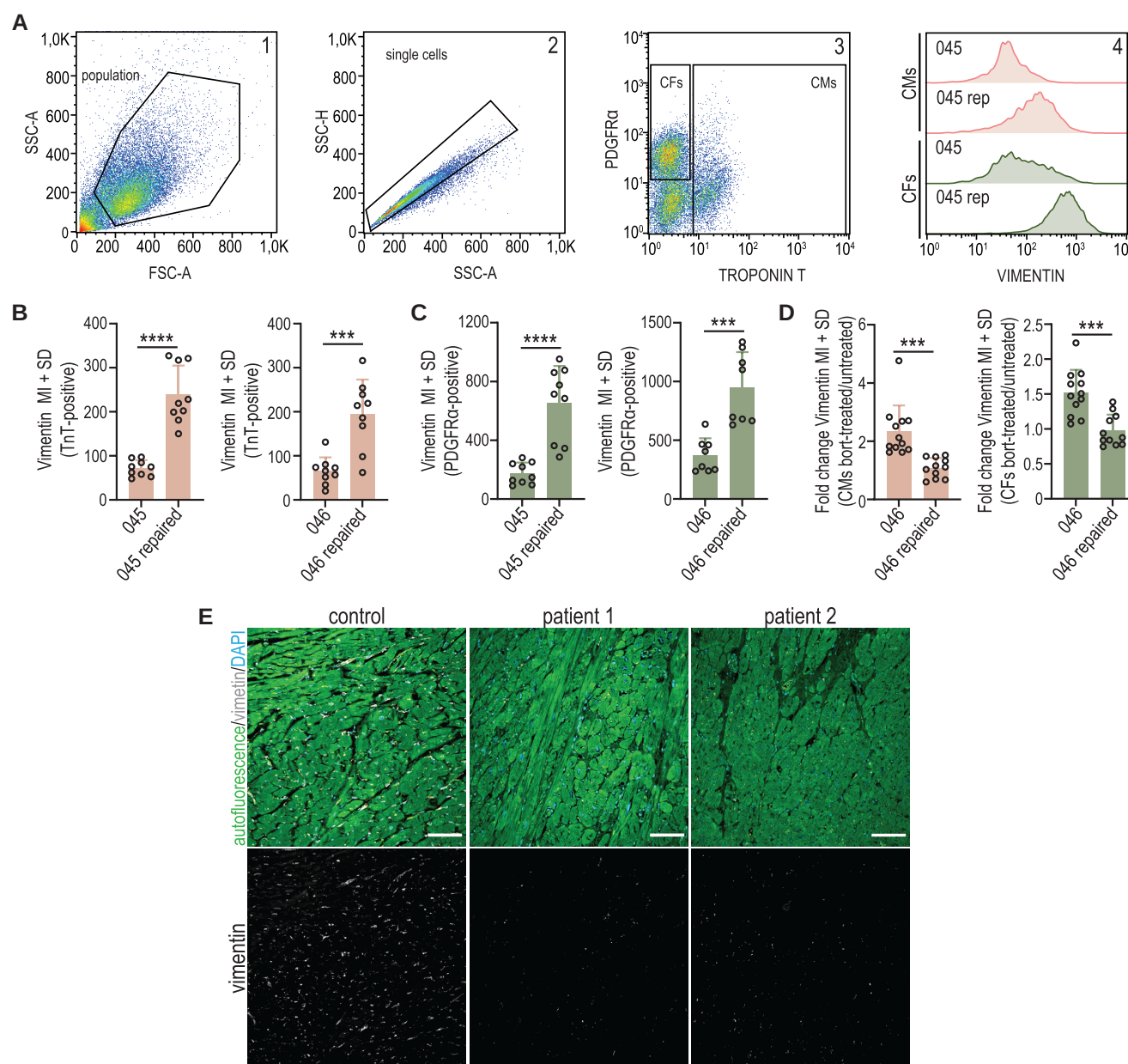


Figure 4 KLHL24 mutation results in increased proteasomal degradation of vimentin in CFs. (A) Gating strategy of the flow cytometry analysis of CM and CF populations isolated from cardioids and representative histograms (right) of the flow cytometry analysis of vimentin in both cell types. PDGF receptor α (PDGFR α) was used as a marker of CFs, and cardiac troponin T as a marker of CMs. (B–C) Quantification of vimentin mean intensity as measured by flow cytometry in CMs (B) and CFs (C) population. Note overall lower levels of vimentin in CMs as compared to CFs. Significantly lower levels of vimentin are observed in 045 and 046 as compared to their isogenic controls [unpaired *t*-test, ****P* < 0.001, *****P* < 0.0001, mean + SD. Each dot represents a sample (*n* = 8–9) consisting of 2–3 merged cardioids. 3 independent differentiation rounds were performed. (D) Quantification of vimentin mean intensity as measured by flow cytometry in CF and CM population isolated from bortezomib (10 nM, 43 h)-treated and untreated cardioids. The increase in mean intensity of vimentin upon treatment is significantly bigger in 046 as compared to 046 repaired CMs and CFs (unpaired *t*-test, ****P* < 0.001, mean + SD. Each dot represents a sample (*n* = 12 (046), 11 (046 repaired)) of merged 2–3 cardioids from 4 independent differentiation rounds. (E) Representative confocal images of LV, showing strongly reduced vimentin levels in tissue slides of patients as compared to the age-matched healthy controls. Muscle bundles were imaged using autofluorescence at a 488 nm wavelength. Scale bar: 100 μ m. Images were counterstained for nuclei (DAPI) and are presented as maximum projected z-stacks.

complexes I, III, IV, mitochondrial ATP synthase, the TIM23 translocase complex, mitochondria solute carrier proteins, the OGDHC-like super-complex, and mitochondrial proteins involved in fatty acid metabolism (such as mitochondrial trifunctional protein and CPT1) (Figure 5C). mRNA expression analysis of subunits of mitochondrial complexes I and

IV revealed no differences between KLHL24^{mut} and control CMs (see [Supplementary material online, Figure S8A](#)), suggesting that the downregulation of mitochondrial proteins in KLHL24^{mut} CMs occurs at the post-translational level. Electron microscopy analysis of mitochondrial structure, number, and cristae organisation revealed no obvious differences between

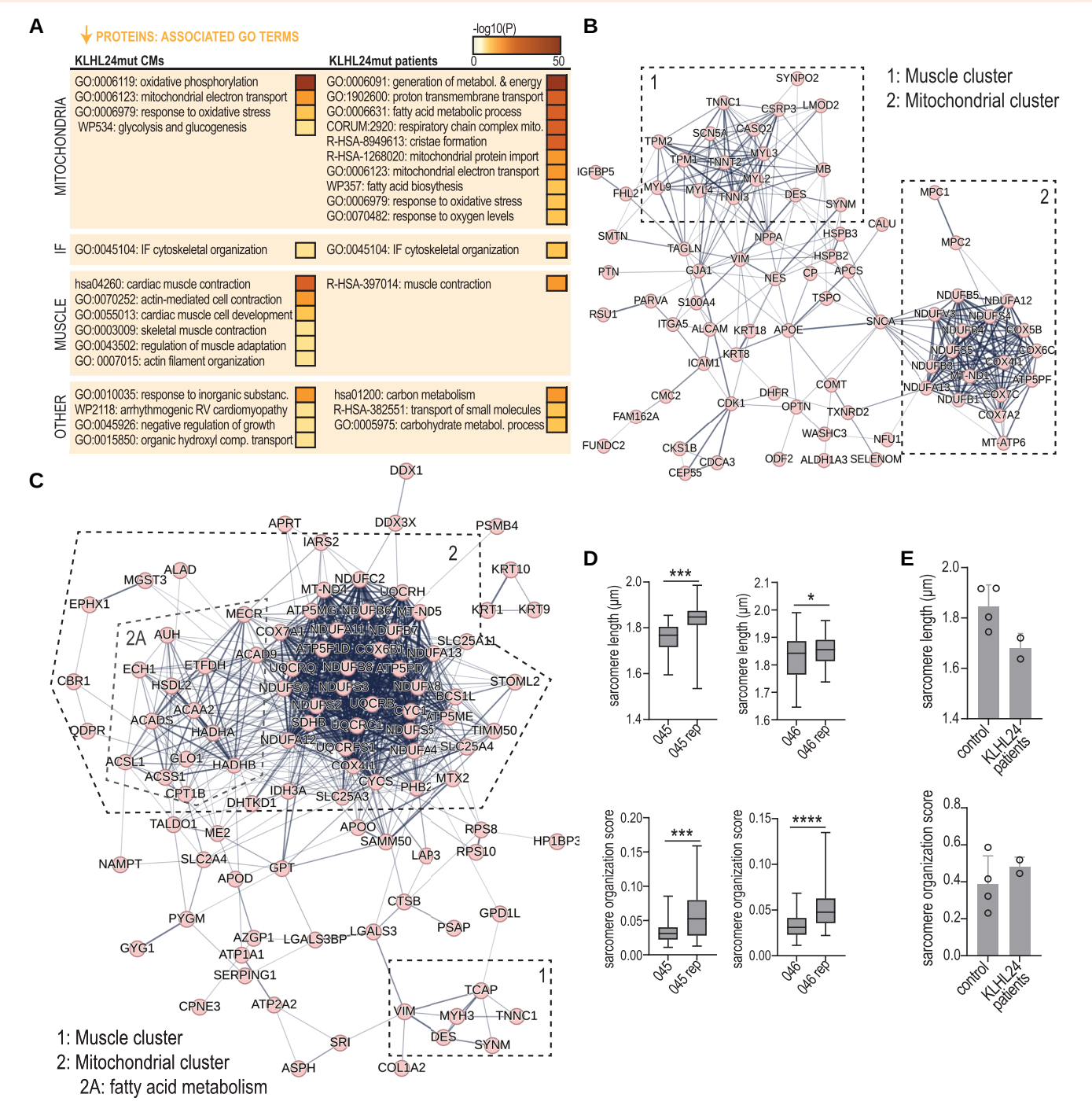


Figure 5 KLHL24 mutation leads to reduced muscle and mitochondrial function. (A) Selected terms from the top 20 enrichment ontology terms associated with proteins reduced in mass spectrometry analysis of 045 compared to 045 repaired hiPSC-derived CMs and of patient compared to control LV tissue sections. For CMs, significantly increased proteins with at least two detected peptides were analysed. For tissue sections, proteins with at least two detected peptides, an abundance ratio variability lower than 80 and with an abundance ratio patient/control <0.6 were analysed. (B–C) STRING interactome network analysis of the group of proteins presented in panel A, calculated for (B) CMs and (C) patient LVs. Prominent muscle (1) and mitochondrial (2) clusters are delineated in both networks. The mitochondrial cluster of the patient data set contains fatty acid metabolism subcluster (2A). (D) Sarcomere quantification analysis of hiPSC-derived CMs (representative images: [Supplementary material online, Figure S7E](#)). Sarcomeres are significantly shorter and show a lower organisation score in 045 and 046 CMs as compared to their isogenic controls. $N = 45$ (045), 43 (045 repaired), 40 (046), 45 (046 repaired) images of 2–3 independent differentiations of hiPSC were used for quantifications (unpaired t-test, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, box and whiskers plots: min—max). (E) Sarcomere quantification analysis of patients' LV CMs (representative images: [Supplementary material online, Figure S7F](#)). Sarcomeres tend to be shorter in patient LV CMs as compared to CMs of control age-matched tissues. Each dot represents an independent sample ($n = 4$ (control), 2 (patients)) and is an average of 15–17 images (mean + SD is shown)).

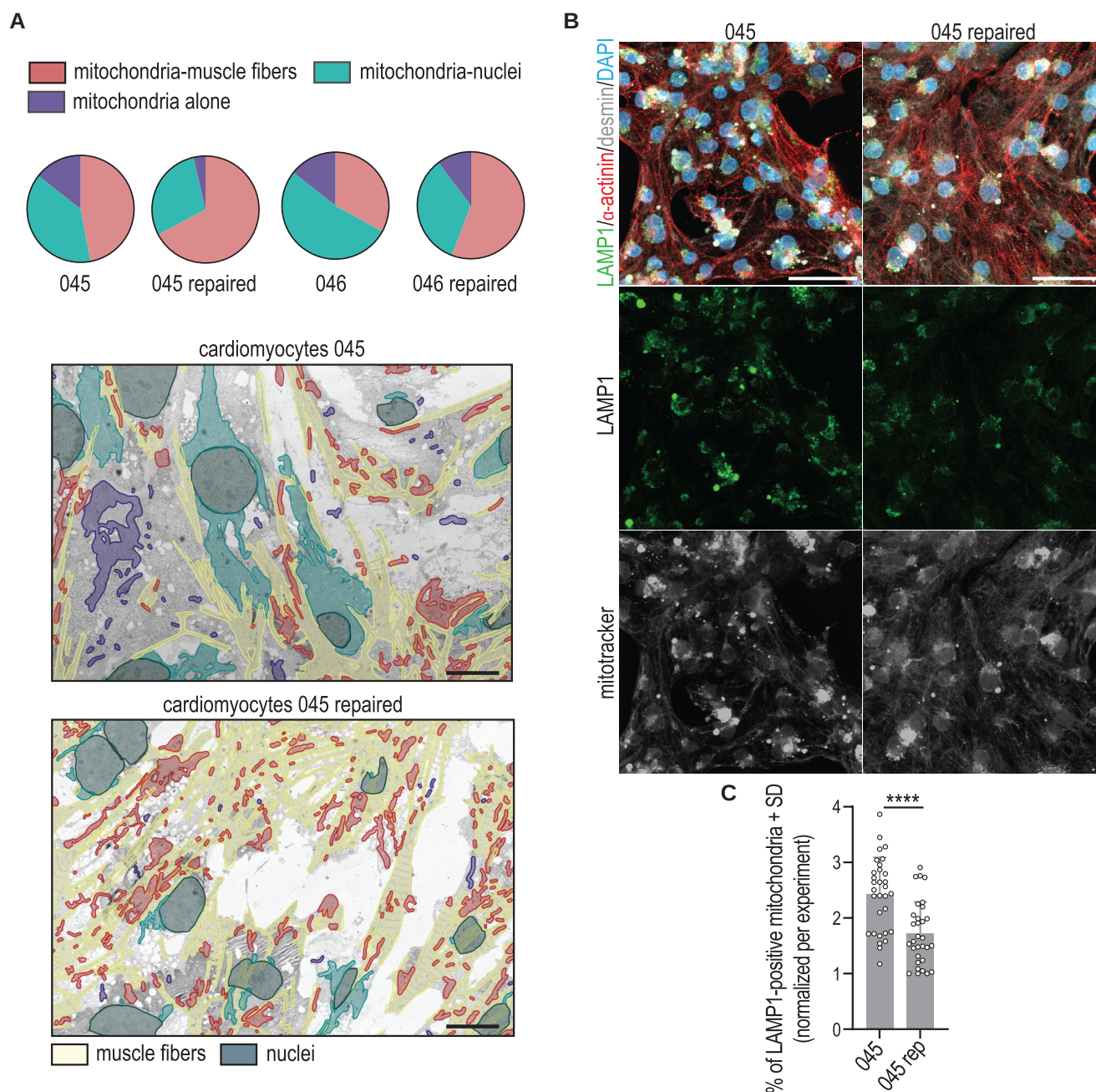


Figure 6 Aberrant mitochondrial localisation and elevated mitophagy in KLHL24^{mut} CMs. (A) Quantification and representative electron microscopy (EM) images of mitochondrial clusters. Note the reduced distribution of mitochondria to muscle fibres in KLHL24^{mut} CMs as compared to their isogenic controls. Uncropped EM stitched tile images used for quantifications can be found in the [Supplementary material online, Figure S9](#). Scale bar: 10 µm. (B) Representative confocal images of fluorescent mitotracker counterstained with the lysosome marker LAMP1 and α-actinin. Images are presented as maximum intensity projected z-stacks. Scale bar: 50 µm. (C) Quantification of panel (B), showing a significantly reduced percentage of mitochondria colocalising with LAMP1 in 045 compared to 045 repaired CMs. Each dot represents a quantified image ($n = 31$, 3 independent hiPSC differentiations were used for quantifications). Data are presented as the percentage of LAMP1-positive mitochondrial area, normalised per experiment, mean + SD, unpaired t-test, **** $P < 0.0001$).

KLHL24^{mut} and control hiPSC-derived CMs (see [Supplementary material online, Figure S9](#)). However, there was a trend towards altered mitochondrial distribution, with reduced localisation of mitochondria to muscle fibres in 045 and 046 CMs compared to their isogenic controls (*Figure 6A*, [Supplementary material online, Figure S9](#)). This may be a downstream effect of IF protein reduction, as desmin is known to regulate mitochondrial levels, function and subcellular localisation in muscle cells.^{28–31}

The levels of mitochondrial and muscle complex proteins are regulated by a balance between proteasomal degradation and autophagy.³² Autophagosome formation depends on the ATG5/ATG12-mediated conversion of LC3B-I into its lipidated LC3B-II form.³² Mass spectrometry analysis revealed a trend toward reduced levels of the autophagy effectors LC3B and GABARAP in KLHL24^{mut} CMs, while the initiation complex proteins remained unchanged (see [Supplementary material online, Table S5](#)).

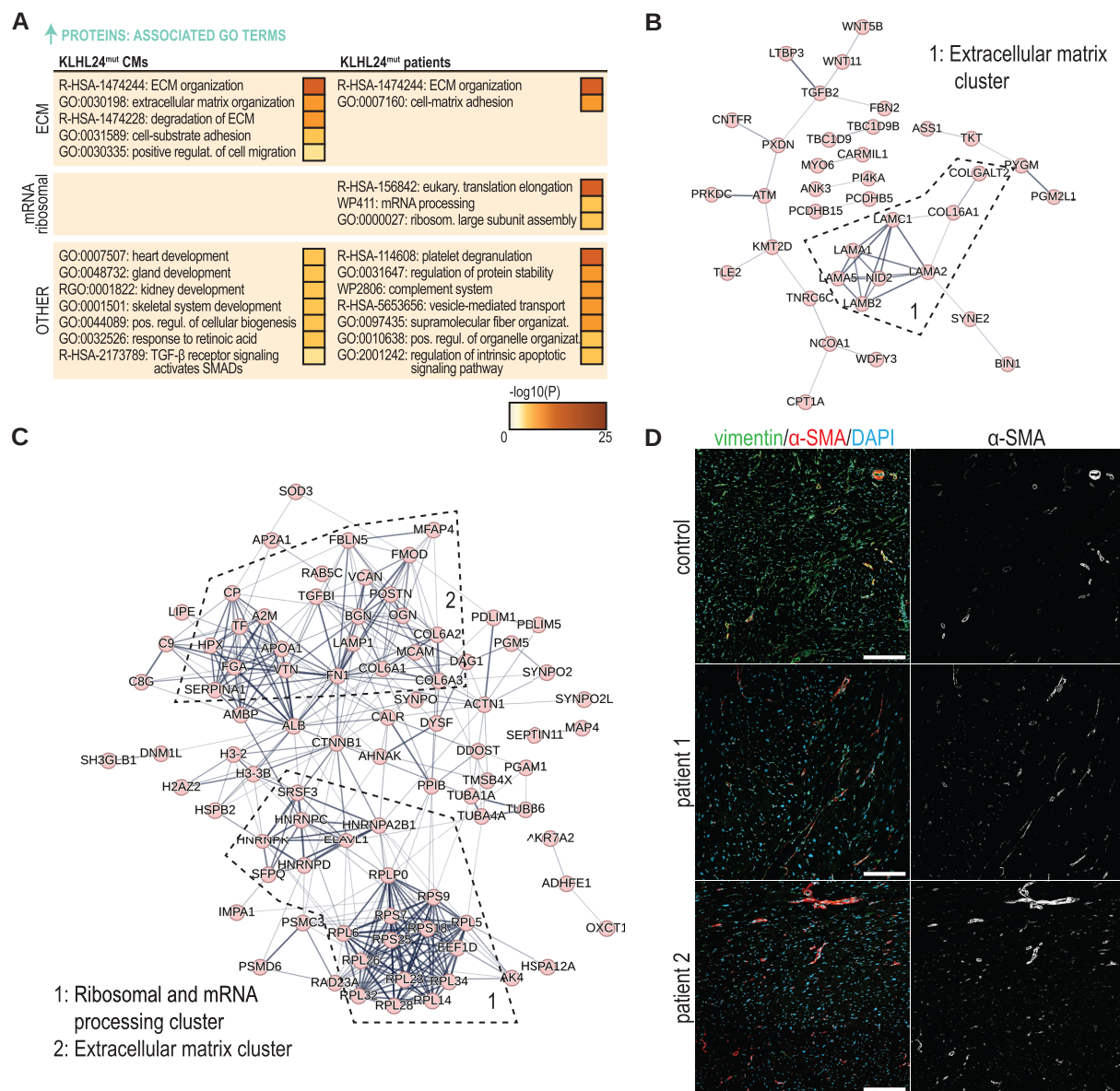


Figure 7 KLHL24 mutation leads to an increased cardiac fibrotic signature. (A) Selected terms from the top 20 enrichment ontology terms associated with proteins that were increased in mass spectrometry analysis of 045 compared to 045 repaired hiPSC-derived CMs and of patient compared to control LV tissue sections. For CMs, significantly increased proteins with at least two detected peptides were analysed. For tissue sections, proteins with at least two detected peptides, an abundance ratio variability lower than 80 and with an abundance ratio patient/control > 1.6 were analysed. (B–C) STRING interactome network analysis of the group of proteins presented in panel B, calculated for (B) CMs and (C) patient LVs. Prominent clusters are delineated: extracellular matrix cluster (B–C) and ribosomal and mRNA processing cluster (C). (D) Representative confocal images showing that α-SMA is abundant in blood vessels and CFs in LV sections of KLHL24 patients. Images are counterstained for vimentin and nuclei (DAPI) and presented as maximum projected z-stacks. Scale bar: 200 μm.

Western blot analysis confirmed decreased levels of LC3B-II in KLHL24^{mut} CMs, with no apparent impairment in the conversion from LC3B-I to LC3B-II (see [Supplementary material online, Figure S8B–C](#)), consistent with comparable levels of ATG complex proteins between KLHL24^{mut} and KLHL24^{WT} CMs (see [Supplementary material online, Table S5](#)). Interestingly, we observed a small but significant increase in AMBRA1 levels, a protein known to play a key role in mitophagy induction³³ (see [Supplementary material online, Table S5](#)). Immunofluorescence analysis showed a higher percentage of mitochondria colocalising with lysosomes in KLHL24^{mut} compared to KLHL24^{WT} CMs, suggesting increased mitophagy associated with KLHL24 mutation (Figure 6B and C).

Taken together, these findings demonstrate that KLHL24^{mut} drives a reduction in numerous proteins critical for the function and physiology of cardiac mitochondria and muscle.

3.7 KLHL24 mutation leads to an increased cardiac fibrotic signature

Enrichment ontology clusters and interactome network analyses of proteins that showed significantly increased abundance in hiPSC-derived CMs in the presence of KLHL24^{mut} revealed relatively few interactions. The most prominent ontology terms were related to extracellular matrix (ECM) and TGF-β receptor signalling (Figure 7A). The interactome network

highlighted a singular cluster containing the major laminin subunits found in the heart, including laminins-221 and -521 (LAMA1, LAMA2, LAMA5, LAMB2, LAMC1) along with other ECM-associated proteins such as nidogen 2 (NID2), fibrillin 2 (FBN2), and a subunit of non-fibrillar collagen type XVI (COL16A1), expression of which has been shown to be elevated in DCM.³⁴ Additionally, the most prominent non-clustered protein that showed increased abundance in KLHL24^{mut} CMs is TGF- β 2 (TGF β 2), an isoform of the master regulator of cardiac fibrosis, TGF- β . TGF- β plays a crucial role in heart development and is a key player in fibrosis processes during cardiac disease^{35,36} (Figure 7B).

In line with observations from monotypic CM cultures, the most prominent ontology term associated with proteins showing increased levels in patient tissue was related to ECM (ECM organisation, cell-matrix adhesion). Additional terms were associated with protein translation (eukaryotic translation elongation, mRNA processing, ribosomal large subunit assembly), protein stability (regulation of protein stability, regulation of intrinsic apoptotic signalling pathway), and vesicle-mediated transport (Figure 7A). Similarly, interactome clustering revealed two distinct clusters: a ribosomal and mRNA processing cluster, and an ECM cluster (Figure 7C). The ribosomal and mRNA processing cluster contained various components of the ribosomal subunits, ribonucleoproteins and RNA-binding proteins. This suggests increased protein synthesis, which is consistent with observations of enhanced protein production during prolonged heart stimulation, such as in heart failure.³⁷ The ECM cluster was centred around fibronectin (FN1), a protein essential for collagen matrix deposition and a major contributor to the increased abundance of cardiac myofibroblasts in the injured heart.³⁸ This finding is in line with the high levels of fibrosis observed in patients (Figure 1). Moreover, adipocyte inclusions observed in patients (Figure 1C) are reflected by increased levels of desensitised lipase, a key enzyme involved in lipid mobilisation and breakdown (see [Supplementary material online, Table S4](#)). Other proteins found in this cluster further reflected cardiac fibrosis, including collagen VI (COL6A1, COL6A2, COL6A3),³⁹ biglycan (BGN), osteoglycin (OGN), versican (VCAN),⁴⁰ periostin (POSTN),⁴¹ and the master-regulator of cardiac fibrosis, TGF- β 1 (TGF β 1).³⁵ Additional proteins in this cluster have been associated with various heart pathologies. For instance, MCAM is linked to cardiac endothelial damage,⁴² fibromodulin (FMOD) to heart failure,⁴³ vitronectin (VTN) to mitochondrial dysfunction⁴⁴ and fibrinogen (FGA) to an increased risk of coronary events.⁴⁵ One particularly notable protein outside these clusters but with a well-known correlation to cardiac fibrosis and myofibroblast conversion of CF is β -catenin (CTNNB1).⁴⁶ Consistent with this, patient CFs exhibited elevated levels of the myofibroblast marker α -smooth muscle actin (α -SMA) (Figure 7D). Such differences were not observed in the hiPSC-derived cardioid CFs, which express α -SMA ubiquitously (see [Supplementary material online, Figure S10](#)). The abundant collagen deposition, upregulation of cardiac fibrotic markers, and myofibroblast activation observed in KLHL24 patients (Figure 1, 7A–D) clearly demonstrate that KLHL24^{mut} promotes the development and progression of cardiac fibrosis. Notably, this mechanism may be tissue-dependent, as KLHL24^{mut} has been shown to reduce wound healing, collagen deposition and myofibroblast activation in dermal fibroblasts.⁹

In conclusion, our study demonstrates that KLHL24^{mut} mediates the excessive degradation not only of desmin, but also of synemin and vimentin. In cardiac tissue, its effects extend beyond CMs or IF proteins, affecting CFs and a wide array of proteins. This leads to impaired PKA signalling, disrupted mitochondrial function and localisation, alterations in autophagy and sarcomere structure, and a pronounced fibrotic signature.

4. Discussion

Although KLHL24 gain-of-function mutations have only been described within the last decade,^{1,2} a recent review of clinical outcomes in KLHL24^{mut} patients already includes 62 cases from 34 families.⁵ While these patients typically present with epidermolysis bullosa at birth, they often develop DCM later in life, after dermatological care is no longer required. On average, 27% of reported KLHL24^{mut} patients develop DCM, with men being at higher risk of earlier onset compared to women.⁵ In the Chilean cohort of seven patients presented in this study, the rate of

cardiac involvement is notably high: both adult males rapidly progressed to heart failure at ages 27 and 34. Of the three adult females (ages 28–38), two experienced a progression of cardiac symptoms over the last five years. This emphasises the necessity for ongoing clinical monitoring of patients beyond their dermatological needs.

Desmin, a key target of KLHL24 in the heart, was previously reported to be reduced in the LV of two KLHL24^{mut} patients, while increased desmin levels were observed in KLHL24 patients with loss-of-function mutation.^{10,18,47} Our study corroborates these findings, showing that KLHL24^{mut} promotes excessive degradation of desmin in CMs at endogenous expression levels. Desminopathy is a known cause of DCM, and reduced desmin levels are associated with increased heart failure in patients with idiopathic DCM,⁴⁸ making desmin a strong candidate as the primary driver of KLHL24^{mut}-mediated DCM and heart failure. Desmin is essential for maintaining muscle fibre integrity, and its loss leads to reduced sarcomere length and defects in the CM contractility.^{31,48,49} The IF network, particularly desmin in CMs and myocytes, also plays a crucial role in mitochondrial function and distribution.^{28–31,48} Mitochondrial defects often precede other abnormalities in CMs and are thought to be the primary driver of desminopathy-related cardiac pathology, leading to CM death, sarcomere structure disruption, altered myofilament interactions, adverse heart remodelling and fibrosis—hallmarks of heart failure.^{48,50} This is consistent with our findings, where we observed significant downregulation of mitochondrial proteins, increased mitophagy, and mislocalization of mitochondria in hiPSC-derived KLHL24^{mut} CMs, where the fibrotic signature remains limited.

However, desmin is not the only downstream target affected by KLHL24^{mut} in CMs. Our study reveals that KLHL24^{mut} promotes the reduction of several proteins, providing a comprehensive list of potential KLHL24 substrates that may contribute to cardiac pathology. Notably, KLHL24^{mut} induces the excessive proteasomal degradation of synemin, an emerging player in skeletal and cardiac myopathies, including DCMs.^{19,20,51} As an IF protein, β -synemin links desmin to Z-disks, while α -synemin stabilises junctional complexes at the sarcolemma.⁵² Desmin and synemin form the IF cytoskeleton in CMs and are highly interconnected, as synemin cannot form stable IFs independently, but binds desmin and/or vimentin to create heterofilaments.²⁰ A limitation of current studies on KLHL24^{mut}, including ours, is the challenge of identifying direct downstream targets of endogenous KLHL24^{mut} due to its low expression levels (e.g. it was undetectable in any of our proteomics screens) and the lack of validated experimental tools such as anti-KLHL24 antibodies. While KLHL24^{mut} downregulates several IF proteins, mitochondrial components, and muscle complex proteins, it remains unclear whether it directly targets multiple proteins or affects a singular target that regulates others. Developing endogenously tagged KLHL24/ KLHL24^{mut} models could provide better mechanistic insights. That being said, our study suggests that KLHL24^{mut} independently targets desmin and synemin for proteasomal degradation. Although both proteins were reduced in the ventricles of KLHL24 patients, high desmin levels in the atria were associated with reduced synemin, while increased synemin expression in KLHL24^{mut} CMs did not lead to elevated desmin levels. This is consistent with previous knock-out and knock-down studies of synemin and desmin, which did not show changes in each other's protein levels,^{52,53} suggesting that they are regulated independently. Furthermore, proteomic analysis of hearts from mice with a neonatal-lethal CM-specific deletion of cullin 3 (the E3 ligase associated with KLHL24) revealed increased levels of synemin and keratin 18, while mitochondrial proteins were mostly reduced, indicating that IF proteins, but not mitochondrial complexes, are likely direct targets of the KLHL24-cullin 3-RING E3 ligase complex.⁵⁴ Interestingly, the patients described in this study show no signs of skeletal muscle pathology, which stands in striking contrast to the clinical picture of desminopathies. This suggests that KLHL24^{mut} differentially affect IF turnover not only in the heart chambers but also possibly in other tissues.

Synemin also functions as a PKA-anchoring protein, thereby modulating key cardiac signalling pathways involved in calcium signalling, excitation–contraction coupling, apoptosis, hypertrophy, and mitochondrial function.^{20,55} Reduced phosphorylation of the transcription factor CREB, a known PKA substrate, indicates impaired PKA activity in KLHL24^{mut}

CMs. Our study, therefore, expands the role of KLHL24^{mut} beyond maintaining structural cell integrity. Impaired PKA signalling in KLHL24^{mut} CMs may also contribute to the observed sarcomere defects. Indeed, sarcomere shortening in KLHL24^{mut} CMs is likely driven by a combination of factors: reduced levels of myofibrillar and cytoskeletal proteins, diminished PKA signalling, and decreased expression of key proteins involved in electrophysiology and excitation-contraction coupling, including Calsequestrin-2 and Calumenin, which regulate Ca²⁺ handling in the sarcoplasmic reticulum, as well as the sodium channel SCN5A. Our findings, therefore, open avenues for further electrophysiological investigations. Intriguingly, despite sarcomere shortening, contraction amplitude was not altered in KLHL24^{mut} CMs when assessed using a 2D model. This is consistent with findings from the van der Meer research group, which showed that KLHL24-related differences in CM contractility become apparent only under conditions of sustained dynamic mechanical load in a complex engineered heart tissue model¹⁰.

Our discovery that CMs are not the only cell type affected in the hearts of KLHL24 patients is significant. We show that KLHL24^{mut} promotes the degradation of vimentin in hiPSC-derived CFs, with severely reduced vimentin levels across all heart chambers of KLHL24^{mut} patients. CFs are essential for cardiac homeostasis and stress response, and contribute to the majority of the ECM deposition, which makes them a key player in cardiac fibrosis.⁵⁶ A recent study on KLHL24^{mut} mice reported reduced vimentin in dermal fibroblasts, which was associated with impaired collagen deposition and fibroblast activation.⁹ This aligns with findings from other studies, showing that vimentin is required for collagen organisation and mesenchymal cell expansion during fibrosis.⁵⁷ Despite low vimentin levels, KLHL24^{mut} patients exhibit extensive fibrosis, myofibroblast activation and upregulation of ECM proteins, particularly in tissue samples reflecting end-stage disease. Interestingly, early fibrotic signatures are already detectable in hiPSC-derived CMs. It remains to be determined whether vimentin loss directly affects CF function in fibrosis or whether the cardiac fibrosis arises solely from CM dysfunction. Alternatively, KLHL24 may increase ECM levels via an independent mechanism, as seen with KLHL12-cullin 3 ligase, which is essential for collagen export and deposition.⁵⁸

Vimentin also plays broader roles in fibroblasts, potentially influencing disease progression by affecting cell velocity, metabolism, or inflammatory responses.⁵⁹ Other cell types, such as the epicardium and endothelial cells, may also contribute to the KLHL24^{mut}-driven cardiac pathology, as IFs are critical for their homeostasis. The epicardium is also a major source of CFs, making it a potential area for future investigation.⁵⁹

Our study utilised both hiPSC-derived cardiac models and patient tissue samples, revealing remarkably consistent findings across these models. This consistency highlights the effectiveness of hiPSC-derived CMs in recapitulating the key pathologies of KLHL24^{mut} cardiac disease, paving the way for future research aimed at drug discovery, including large-scale drug screens and the identification of novel therapeutic compounds.

In conclusion, patients with KLHL24 gain-of-function mutations are at high risk of developing DCM in adulthood. Our study shows that KLHL24^{mut} promotes excessive proteasomal degradation of desmin and synemin, impairs PKA signalling, leads to reduced levels of several critical mitochondrial and muscle proteins in CMs and drives extensive fibrosis. Importantly, we found that CMs are not the only cardiac cell type affected; KLHL24^{mut} also induces excessive degradation of vimentin in CFs, compromising their mechanical stability. Furthermore, our hiPSC-derived CM model recapitulates the key cardiac pathologies observed in KLHL24^{mut} patients and is thus a physiologically relevant tool for future drug-discovery studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

VR designed, performed and analysed the experiments and wrote the manuscript. KR designed the experiments and wrote the manuscript. SC,

MJ and LS analysed clinical data, and SC and MJ wrote the clinical part of the manuscript. AP performed cardioid experiments, RT and PvV performed and analysed mass spectrometry experiments, and AM and RK performed TEM experiments. Chu, RI-A, RdO, AS, MJY and FP provided patient care. BM, CP, IF and PM provided patient samples. IF and PM provided clinical data and samples for the generation of hiPSC lines and CH, IF, MJ, CLM and KR provided discussion and CLM and KR provided supervision. CH, IF, CLM and KR conceived the study. All authors read and approved the manuscript.

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Conflict of interest: None declared.

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Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁶⁰ partner repository with the dataset identifier PXD051917.

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