Received: 2 October 2018 | Revised: 25 January 2019 | Accepted: 17 February 2019

DOI: 10.1002/humu.23728

RESEARCH ARTICLE

WILEY Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY

Functional characterization of *CHEK2* variants in a *Saccharomyces cerevisiae* system

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Funding information
A.G. Leventis Foundation; IKY_Greek State Scholarships Foundation; research program ARISTEIA, Grant/Award Numbers: P. BROCA, ARISTEIA 39; European Union (European Social Fund, ESF); Greek national funds through the Operational Program; Target Identification and Development of Novel Approaches for Health and Environmental Applications, Grant/Award Number: MIS 5002514; Competitiveness, Entrepreneurship and Innovation, Grant/Award Number: NSRF 2014–2020; Greece and the European Union (European Regional Development Fund)

Abstract

Genetic testing for cancer predisposition leads to the identification of a number of variants with uncertain significance. To some extent, variants of *BRCA1/2* have been classified, in contrast to variants of other genes. *CHEK2* is a typical example, in which a large number of variants of unknown clinical significance were identified and still remained unclassified. Herein, the *CHEK2* variant assessment was performed through an in vivo, yeast-based, functional assay. In total, 120 germline *CHEK2* missense variants, distributed along the protein sequence, and two large in-frame deletions were tested, originating from genetic test results in breast cancer families, or selected from the ClinVar database. Of these, 32 missense and two in-frame deletions behaved as non-functional, 73 as functional, and 15 as semi-functional, after comparing growth rates of each strain with positive and negative controls. The majority of non-functional variants were localized in the CHK2 kinase and forkhead-associated domains. In vivo results from the non-functional variants were in agreement with in silico predictions, and, where available, with strong breast cancer family history, to a great extent. The results of the largest, to date, yeast-based assay, evaluating *CHEK2* variants, can complement and assist in the classification of rare *CHEK2* variants with unclear clinical significance.

KEYWORDS
breast cancer, *CHEK2* variants, functional assay, yeast

*These authors jointly supervised this work.

1 | INTRODUCTION

In the context of multigene testing for cancer predisposition, a significant number of variants of uncertain clinical significance (VUS), are frequently detected. Although collaborative groups of experts (e.g., Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA)-consortium) have been working for many years toward the classification and clinical significance of VUS in *BRCA1* & *BRCA2*, employing data from families in functional assays, VUS classification in other genes is quite precarious. Of those, *CHEK2* VUS are of particular interest, mainly due to the plethora of such variants identified, as well as to the association of *CHEK2* damaging alleles with an increased risk of various types of cancer. More specifically, definitely pathogenic *CHEK2* variants predispose to breast, colon, prostate, thyroid, and kidney cancer, with the risks being variable (CHEK2 Breast Cancer Case-Control Consortium, 2004; Cybulski et al., 2004; Dong et al., 2003; Kilpivaara, Alhopuro, Vahteristo, Aaltonen, & Nevanlinna, 2006; Siolek et al., 2015). Up to date and based on ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), 675 unique *CHEK2* missense variants have been reported.

CHEK2 is a tumor suppressor gene, encoding a serine-threonine kinase (CHK2), which plays an important role in genomic integrity and cellular response to DNA damage, through phosphorylation of several substrates, including BRCA1, p53, Cdc25A and Cdc25C phosphatases, E2F-1 transcription factor, and promyelocytic leukemia protein (Falck, Mailand, Syljuasen, Bartek, & Lukas, 2001; Hirao et al., 2000; J. S. Lee, Collins, Brown, Lee, & Chung, 2000; Peng et al., 1997; Stevens, Smith, & La Thangue, 2003; Yang, Kuo, Bisi, & Kim, 2002). CHK2 protein consists of three distinct domains: the SQ/TQ cluster domain (SCD) at the N-terminus, the central forkhead-associated (FHA) region, and the kinase domain, which occupies a significant part of the C-terminal end. There is also a nuclear localization signal (NLS) domain at the C-terminus. SCD domain serves as a regulatory region that interacts with ataxia-telangiectasia mutated (ATM) and ATR (ATM- and Rad3-related) kinases in response to DNA double-strand breaks, FHA domain mediates protein-protein interactions, whereas the kinase region is the catalytic domain (Bartek, Falck, & Lukas, 2001).

The involvement of *CHEK2* gene in cancer predisposition was first demonstrated with the identification of the c.1100delC mutation in families with multiple cancers (Bell et al., 1999), resulting in abrogation of the kinase activity (Wu, Webster, & Chen, 2001). This specific loss-of-function allele has been associated with increased breast cancer risk since 2002 (Meijers-Heijboer et al., 2002). More recent studies report a two- to three-fold increase in breast cancer risk in women carrying the c.1100delC or other *CHEK2* truncating variant and a 3.8-fold increase in men (Couch et al., 2017; Decker et al., 2017; Hauke et al., 2018; Pritzlaff et al., 2017; Slavin et al., 2017).

However, the association of missense *CHEK2* variants with breast cancer is far from being clear. The relatively frequent missense variant p.(Ile157Thr), which is located within the FHA domain, is possibly the most controversial in terms of pathogenicity. Such examples highlight the need for utilization of additional tools that can be of assistance to the variant classification process.

In an effort to classify emerged *CHEK2* variants, we have assessed the functional consequences of a number of them in an in vivo yeast-based assay. In total, 120 germline *CHEK2* missense variants, distributed along the protein sequence, and two large in-frame deletions, have been selected to be analyzed. These have been detected in Greek breast cancer families, proposed by members of the ENIGMA consortium, or selected from the ClinVar database (variants with multiple submissions from various ethnicities were preferred). According to their DNA repairability, after chemically induced DNA damage, all tested *CHEK2* variants have been categorized in three distinct groups, namely, benign (functional), intermediate (semi-functional), and damaging (non-functional). The results of our assay can complement and assist in the classification of relatively rare *CHEK2* variants with unclear clinical significance.

2 | MATERIALS AND METHODS

2.1 | Yeast strain and plasmids

The *Saccharomyces cerevisiae* strain used was W2105-17b (MATa sml1Δ::URA3 rad53Δ::HIS3 RAD5 leu2–3, 112 trp-1-1 can1–100 ura3-1 ade2-1 his3–11,15), which lacks *RAD53*, the yeast homolog of human *CHEK2*, as well as the *SML1* gene, encoding an inhibitor of ribonucleotide reductase (kindly provided by Dr. Rodney Rothstein, Department of Genetics and Development, Columbia University Medical Center). *SML1* deletion results in increased levels of all four dNTPs for DNA synthesis (Zhao et al., 2000).

All missense variants to be tested, and c.1100delC mutation, were introduced into a yeast expression plasmid by site-directed mutagenesis using the Q5 Site-Directed Mutagenesis kit (NEB, Ipswich, MA), following the manufacturer's instructions. The plasmid used was pmh267 (pBAD101, 2 μm LEU2 GAL-CHEK2; Matsuoka, Huang, & Elledge, 1998), which carries the wild type *CHEK2* (kindly provided by Dr. Stephen Elledge, Center of Genetics and Genomics, Harvard Medical School).

Generation of competent yeast cells and transformation with the aforementioned plasmid were carried out using the Frozen EZ Yeast Transformation kit by Zymo Research (Irvine, CA), following the manufacturer's instructions.

2.2 | Sequencing analysis

All variants to be tested were verified by Sanger sequencing of full-length *CHEK2* gene in the construct. Polymerase chain reaction (PCR) products were electrophoresed on an ABI Prism Genetic Analyzer and sequenced using the BigDye Terminator Cycle Sequencing kit (v.3.1; Applied Biosystems, Foster City, CA). The resulting sequences were aligned against *CHEK2* wild-type sequence (GenBank *CHEK2*: NM_007194.3). PCR conditions and sequences of the primers used are available upon request.

2.3 | Yeast functional assay

Transformed yeast cells were grown in His-Ura-Leu plates with 2% glucose for 2–3 days. Single colonies picked from each plate were inoculated into His-Ura-Leu liquid media containing 1% galactose and 1% raffinose, instead of glucose, and incubated at 30°C with shaking at 250 rpm.

DNA damage was provoked by methyl-methanesulfonate (MMS; Sigma-Aldrich, St. Louis, MO), an alkylating agent, which induces replication fork stalling. The underlying idea is that, after degradation of MMS, yeast strains that carry wild-type CHK2 can repair MMS-induced DNA damage and resume cell growth and proliferation, while strains with an abnormal CHK2 protein are incapable of completing DNA replication and entering mitosis, so their population remains numerically stable.

When yeast cultures reached late-log phase, they were first diluted to an optical density of 0.500 ± 0.001, then diluted 10-fold, and transferred to a 96-well plate containing MMS in fresh His-Ura-Leu liquid medium, to a final concentration of 0.00125%. The plates were then incubated at 30°C and 200 rpm, for 22 hr. The conditions were selected after optimization, taking into consideration the half-life of MMS, its prohibitive concentration and the possible culture evaporation after long incubation. For each variant, three independent experiments, each with four to six replicates, were conducted. Characterization of each variant derived from comparisons between its individual mean growth level and the mean growth levels of a negative control strain (c.1100delC) and wild-type *CHEK2* strains at 22 hr. The spectrophotometer used was a HITACHI 150-20 (Hitachi Ltd., Tokyo, Japan).

2.4 | Prediction tools

All variants were in silico tested with PROVEAN-SIFT (http://provean.jcvi.org/index.php; Choi, Sims, Murphy, Miller, & Chan, 2012; Ng & Henikoff, 2001), Align GVGD (updated 2014, http://agvgd.hci.utah.edu/agvgd_input.php; Mathe et al., 2006), PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/; Adzhubei et al., 2010), MutationTaster (http://www.mutationtaster.org/; Schwarz, Cooper, Schuelke, & Seelow, 2014), PhastCons (Siepel et al., 2005), PhyloP (Pollard, Hubisz, Rosenbloom, & Siepel, 2010), GERP (Cooper et al., 2005; Davydov et al., 2010), and Grantham (Grantham, 1974), where applicable, and the minor allele frequency (MAF) of each variant was monitored (http://evs.gs.washington.edu/EVS/). Results of PhastCons and PhyloP were extrapolated from MutationTaster, whereas those of GERP and Grantham, from the Exome Variant Server (http://evs.gs.washington.edu/EVS/). The in silico programs were used by the default settings and parameters. The two LGRs were evaluated with MutationTaster and PROVEAN.

In parallel, the PyMOL software (version 1.7.4.5.; https://pymol.org/2/) was used to predict structural and functional consequences of each variant, while Human Splicing Finder (HSF; version 3.1) was used to predict possible splicing effects (http://www.umd.be/HSF3/; Desmet et al., 2009).

2.5 | Statistical analysis and categorization

For comparing the growth rates of constructs versus those of positive and negative controls, two metrics of the effect size were taken into account, that is, the normalized median and the normalized standardized difference of means (Appendix S2).

All *CHEK2* variants tested were categorized in three groups, namely, benign (functional), intermediate (semi-functional), and damaging (non-functional). For a variant to be categorized as benign or damaging, both the normalized median and the normalized standardized difference of means should fulfill some criteria. Specifically, if the normalized median of a variant is above 0.70 and its normalized standardized difference of means is above 0.30, then the variant is categorized as benign, whereas if the normalized median of a variant is below 0.30 and its normalized standardized difference of means is below −0.30, then the variant is categorized as damaging. Intermediate variants correspond to normalized medians between 0.30 and 0.70 or/and normalized standardized difference of means between −0.30 and 0.30 (results in detail are summarized in Table S1, while a graphical representation of the normalized standardized difference of means is shown in Figure 5).

2.6 | Ethical compliance

Written informed consent was signed by all patients before genetic testing. The studies, which were involved, were approved by the Bioethics Committee of the National Center for Scientific Research "Demokritos" (reference number BC 14/02/2014), in agreement with the 1975 Helsinki statement, revised in 1983.

3 | RESULTS

3.1 | In vivo results

In total, 120 *CHEK2* missense variants, located throughout the coding region of *CHEK2*, including the three critical CHK2 domains (SQ/TQ cluster, FHA, and kinase domains), the NLS, as well as the intermediate regions, were analyzed (variants' position is depicted in Table 1). Of the selected missense variants, 114 have an entry on ClinVar database. The vast majority of them (n = 90) were classified as of uncertain significance, 20 variants had conflicting interpretations of pathogenicity, two and one were classified as likely pathogenic and likely benign, respectively, while one was classified as benign, by ClinVar. A summary of the variant classification based on ClinVar is summarized in Table S3. Variants that were characterized as likely benign or likely pathogenic by ClinVar were included in our series mainly due to their clinical interest and based on the fact that have been detected in Greek cancer patients and families. Novel variants have been submitted in the Leiden Open Variation Database.

Variants with growth comparable to the positive and negative control were characterized as benign and damaging, respectively.

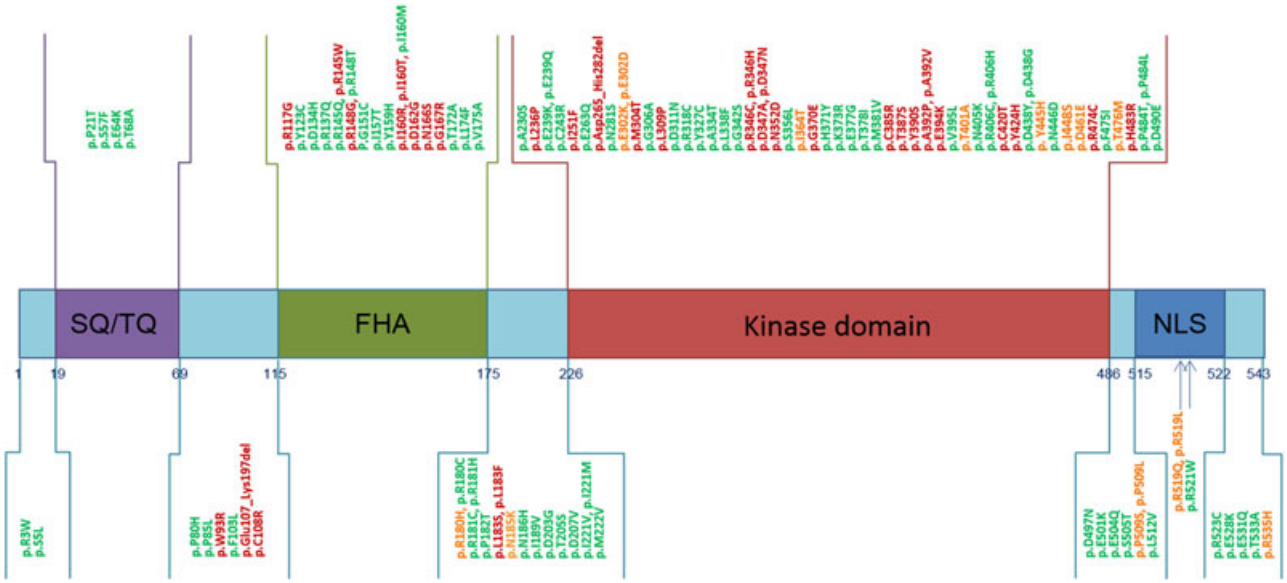

FIGURE 1 *CHEK2* variant characterization and their domain location. Benign variants have been marked in green color, intermediate variants in orange, and damaging variants in red. *The single-letter amino acid code was used and the parenthesis was omitted, herein, due to space restrictions [Color figure can be viewed at wileyonlinelibrary.com]

Variants showing significantly different growth from both positive and negative controls were characterized as intermediate.

Based on the two effect size metrics results, 73 missense variants were categorized as benign, 15 as intermediate, and 32 as damaging. The p.(Glu107_Lys197del) and p.(Asp265_His282del) large in-frame deletions, resulting in ~6 kb deletion of exons 2 and 3 and a ~7 kb deletion of exon 6, respectively, were also categorized as damaging, consistent with prediction from in silico tools and data from families, in which they were detected (Apostolou et al., 2018). A color-coded, graphical representation of the variants tested is shown in Figure 1. Of the variants characterized as damaging, the majority was located within the kinase domain [20 variants, namely, p.(Leu236Pro), p.(Glu302Lys), p.(Met304Thr), p.(Leu309Pro), p.(Arg346Cys), p.(Arg346His), p.(Asp347Asn), p.(Asp347Ala), p.(Asn352Asp), p.(Gly370Glu), p.(Cys385Arg), p.(Thr387Ser), p.(Tyr390Ser), p.(Ala392Pro), p.(Ala392Val), p.(Glu394Lys), p.(Cys420Thr), p.(Tyr424His), p.(Arg474Cys), and p.(His483Arg)], eight variants within the FHA domain [p.(Arg117Gly), p.(Arg145Trp), p.(Arg148Gly), p.(Ile160Arg), p.(Ile160Thr), p.(Asp162Gly), p.(Asn166Ser), and p.(Gly167Arg)], two in the interspace between kinase and FHA domains [p.(Leu183Ser) and p.(Leu183Phe)], and two [p.(Trp93Arg) and p.(Cys108Arg)] in the interspace between SCD and FHA domains.

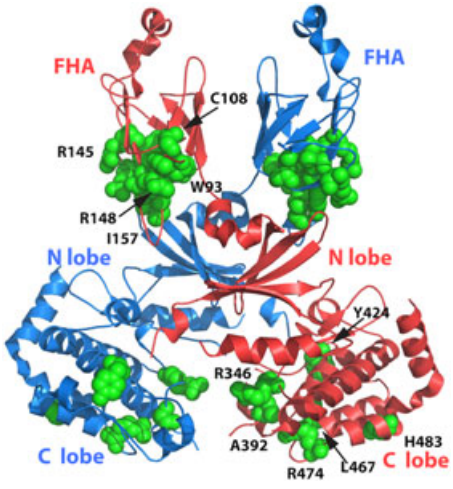

FIGURE 2 Schematic representation of the crystal structure of the CHK2 dimer (from Protein Data Bank, code: 3I6U). One monomer is shown in blue and one on red. The forkhead-associated domain and kinase N-lobe and C-lobe domains are indicated. The mutated residues are indicated by green spheres. *The single-letter amino acid code was used and the parenthesis was omitted, herein, due to space restrictions [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | In silico predictions

CHEK2 variants that were categorized as damaging by our yeast functional assay were in concordance with the majority of the in silico tools predictions obtained in this work. More specifically, for the following variants: p.(Trp93Arg), p.(Cys108Arg), p.(Arg145Trp), p.(Ile160Arg), p.(Ile160Thr), p.(Asp162Gly), p.(Asn166Ser), p.(Leu183Ser), p.(Leu236Pro), p.(Leu309Pro), p.(Arg346Cys), p.(Asp347Ala), p.(Cys385Arg), p.(Tyr390Ser), p.(Ala392Val), p.(Glu394Lys), and p.(Cys420Thr), in silico and functional assay results were in complete concordance. On the other hand, a single discrepancy was noted for the following variants: p.(Arg117Gly), p.(Arg148Gly), p.(Gly167Arg), p.(Leu183Phe), p.(Met304Thr), p.(Arg346Cys), p.(Asp347Asn), p.(Gly370Glu), p.(Thr387Ser), p.(Ala392Pro), p.(Tyr424His), p.(Arg474Cys), p.(His483Arg), and p.(Asn352Asp), while two discrepancies in the in silico tools were noted for the p.(Ile251Phe) variant.

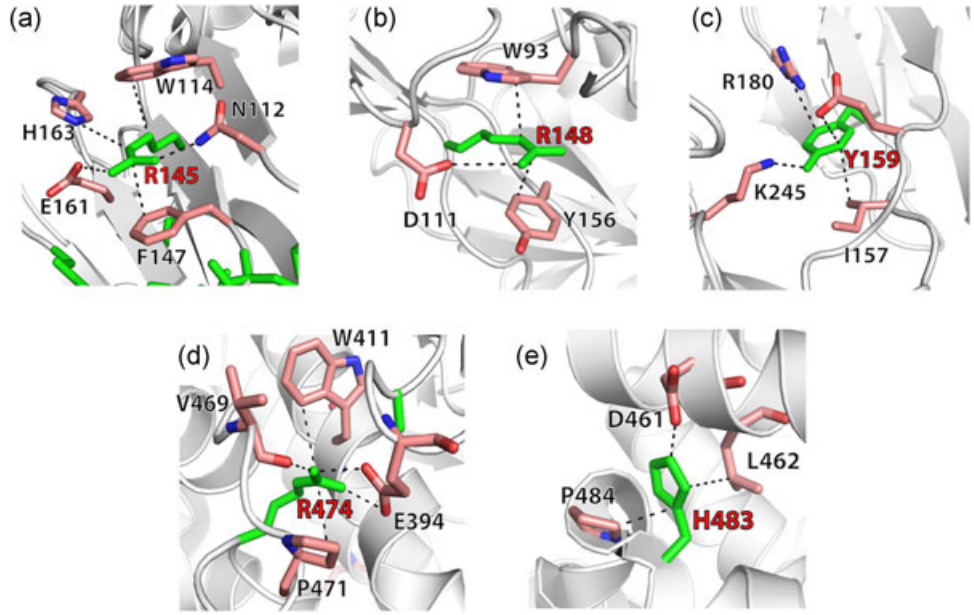

FIGURE 3 Schematic representation of atomic interactions important for local protein stability that may be perturbed by mutation. (a–e) Key interactions of residues Arg145, Arg148, Tyr159, Arg180, and His483. *The single-letter amino acid code was used and the parenthesis was omitted, herein, due to space restrictions [Color figure can be viewed at wileyonlinelibrary.com]

On the contrary, variants p.(Tyr159His), p.(Asp203Gly), p.(Tyr327Cys), p.(Ala334Thr), p.(Gly342Ser), p.(Glu377Gly), and p.(Arg521Trp), which were categorized as benign by the yeast functional assay, were predicted as damaging and/or possibly damaging by in silico predictions (detailed results are shown in Table 1).

Furthermore, the possible splicing effect of the tested missense variants was interrogated by the HSF program, through which, 51 variants were predicted to affect splicing since they displayed high splice site scores (Table S2). The variants predicted to have a possible splicing effect involved damaging (n = 14), intermediate (n = 10), and benign (n = 27) variants, characterized as damaging by the yeast functional assay, but their actual effect in the canonical splicing process should be assessed experimentally.

3.3 | Comparison of results with available functional data

In total, four functional studies assessing *CHEK2* variants have been published. More, specifically 26 variants (Roeb, Higgins, & King, 2012), four variants (Bell et al., 2007), three variants (S. B. Lee et al., 2001), and two variants (Tischkowitz et al., 2008) have been assessed within these studies.

We have, therefore, performed a direct comparison of the results of our assay with the ones previously reported. Among the 17 common tested variants with Roeb et al., there were seven concordant classifications, namely, p.(Pro85Leu), p.(Arg117Gly), p.(Arg137Gln), p.(Arg145Trp), p.(Gly167Arg), p.(Asp347Ala), and p.(Tyr424His). The latter was the only common variant tested by us and Tischkowitz et al., which was characterized as benign and was in disagreement with both our results and those of Roeb et al. Interestingly, there was complete concordance for the three common tested variants [p.(Arg3Trp), p.(Arg145Trp), and p.(Ile157Thr)] between our study and the study by Lee et al., while concordance for a single variant [p.(Arg137Gln)] was among the three common tested variants between our study and the study by Bell et al. Results are summarized in Table 3.

3.4 | Prediction of structure and functional consequences

To gain insight into the functional repercussions and pathogenic potential of the variants tested, as well as to perform an indicative check, we randomly chose 16 variants, assuring that these were located in different protein domains (FHA and kinase domain), as well as in interspaces, to map their location onto the crystal structure of the CHK2 dimer (Protein Data Bank code 3I6U; Cai, Chehab, & Pavletich, 2009). Of these, 14 were selected out of the 32 missense variants that were characterized as damaging, while two were characterized as benign variants, but were chosen based on their significance in clinical interest. The positions of the changed amino acids are shown in Figure 2, mapped onto the CHK2 dimer. Notably, the amino acids that correspond to chosen damaging variants are not randomly distributed onto the structure, but rather cluster around two structural regions: i) in the FHA domain, near the interface with the N-lobe of the kinase domain of the opposing monomers (amino acids changes: 93, 108, 145, 148, 160, and 183), and ii) in the C-lobe of the kinase domain, mapping near the active site and the dimerization interface, that includes the activation loops (Cai et al., 2009). Analysis of the potential implications of the missense variants on the function of the enzyme revealed that most of them can

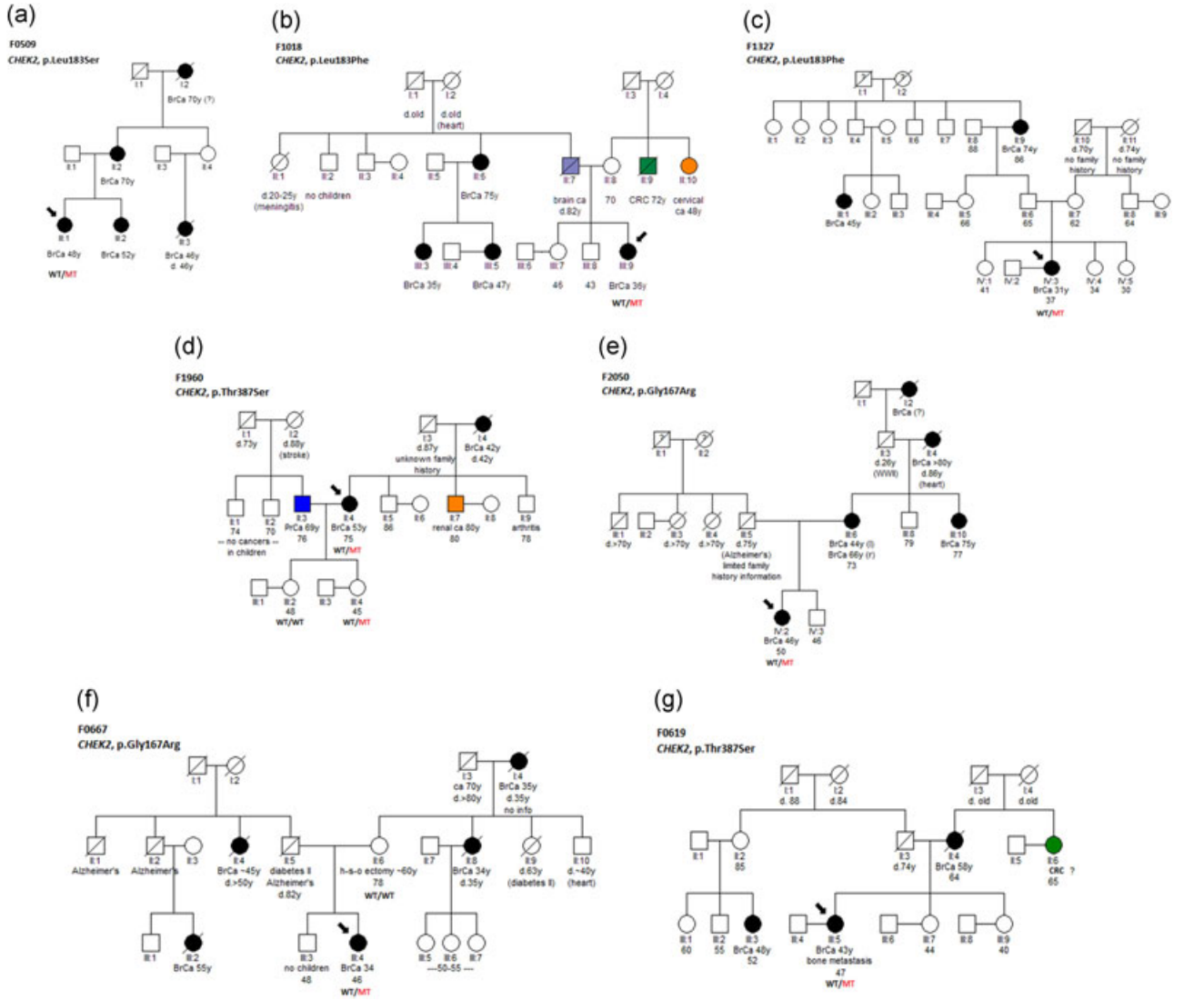

FIGURE 4 Pedigrees of breast cancer families carry damaging *CHEK2* variants. (a) *CHEK2* p.Leu183Ser, (b) *CHEK2* p.Leu183Phe, (c) *CHEK2* p.Leu183Phe, (d) *CHEK2* p.Thr387Ser, (e) *CHEK2* p.Gly167Arg, (f) *CHEK2* p.Gly167Arg, (g) *CHEK2* p.Thr387Ser. Probands are represented by the arrow, while breast cancer patients are colored in black. Breast cancer (BrCa), colorectal cancer (CRC), prostate cancer (PrCa), cancer (ca), carriers of mutations (WT/MT), wild type for the familial mutation (WT/WT) [Color figure can be viewed at wileyonlinelibrary.com]

destabilize key structural interactions within each monomer (Figure 3), therefore affecting thermodynamic stability and/or folding efficiency, both of which can affect the functional protein levels in the cell. In addition, both variant clusters are nearby the dimerization domain or directly participate in dimer formation, and therefore could affect the ability of the kinase to dimerize and be activated. Particular effects of the chosen variants on the structure of the protein are described in Table 2.

On the other hand, the contrast between the effect predictions of structural model for variants p.(Ile157Thr) and p.(Tyr159His), and the benign characterization by the yeast assay, shows that the structural models, as well as the in silico tools, which are mainly based on position conservation, give only an indication of the effect of an amino acid change, and highlights the need of a combined evaluation approach where functional assays are an essential part.

3.5 | Segregation and/or association of classified variants in Greek breast cancer families

Family history information from Greek breast cancer families harboring *CHEK2* missense variants characterized as damaging were combined and correlated with our in vivo assay results to further elucidate the variants' clinical significance. This involved a total of seven families, members of which carry four distinct *CHEK2* variants, namely, p.(Leu183Phe), p.(Leu183Ser), p.(Thr387Ser), and p.(Gly167Arg). As illustrated in Figure 4, probands were diagnosed with breast cancer at a relatively young age, while all cases had a family history of breast cancer among close relatives.

More specifically, although segregation analysis was not feasible, F0509 carries a strong burden of breast cancer, affecting five members distributed in three successive generations.

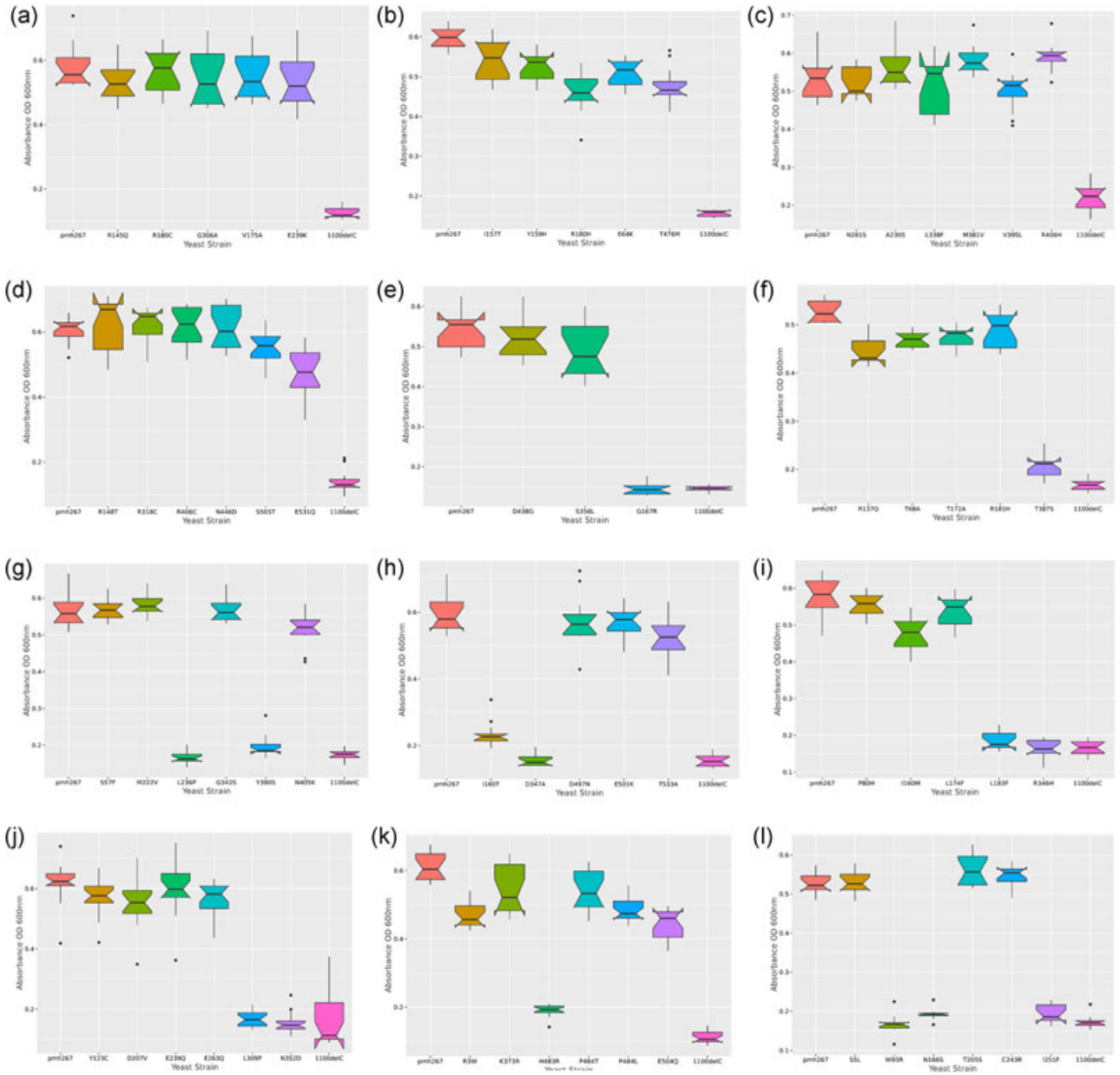

FIGURE 5 Notched boxplot diagrams showing the absorbance at 600 nm for each *CHEK2*-tested variant. Notches represent 95% confidence interval of the median. Each figure corresponds to an independent set of experiments. pmh267: positive control, strains carry the wild type CHK2. c.1100delC: negative control, strains that carry the pathogenic mutation c.1100delC. *The single-letter amino acid code was used and the parenthesis was omitted, herein, due to space restrictions [Color figure can be viewed at wileyonlinelibrary.com]

Moreover, probands of F1018 and F1327, both carrying the p.(Leu183Phe) variant, have been diagnosed with breast cancer at a very young age (that is, 36 and 31 years, respectively). Interestingly, although both families are characterized by multiple breast cancer diagnoses, a family member has been diagnosed with colorectal cancer, possibly associated with *CHEK2* damaging variants.

The p.(Thr387Ser) variant was detected in families F1960 and F0619. In F1960, both the proband and her mother were diagnosed with breast cancer, while one of her two daughters, although found to carry the damaging allele, is cancer-free at the age of 45 years. Among the family members of F0619, three breast cancers have emerged, two of which are early onset, while an additional possible colorectal cancer case from the mother's side, was reported.

Furthermore, the probands of F2050 and F0667 families were found to carry the p.(Gly167Arg) variant. In family F2050, five breast cancer cases were observed in four successive generations, all originating from the proband's mother side. On the other hand, in family F0667, which includes two independent breast cancer cases, the proband inherited the damaging variant from her father, as genotyping of the mother revealed absence of this allele.

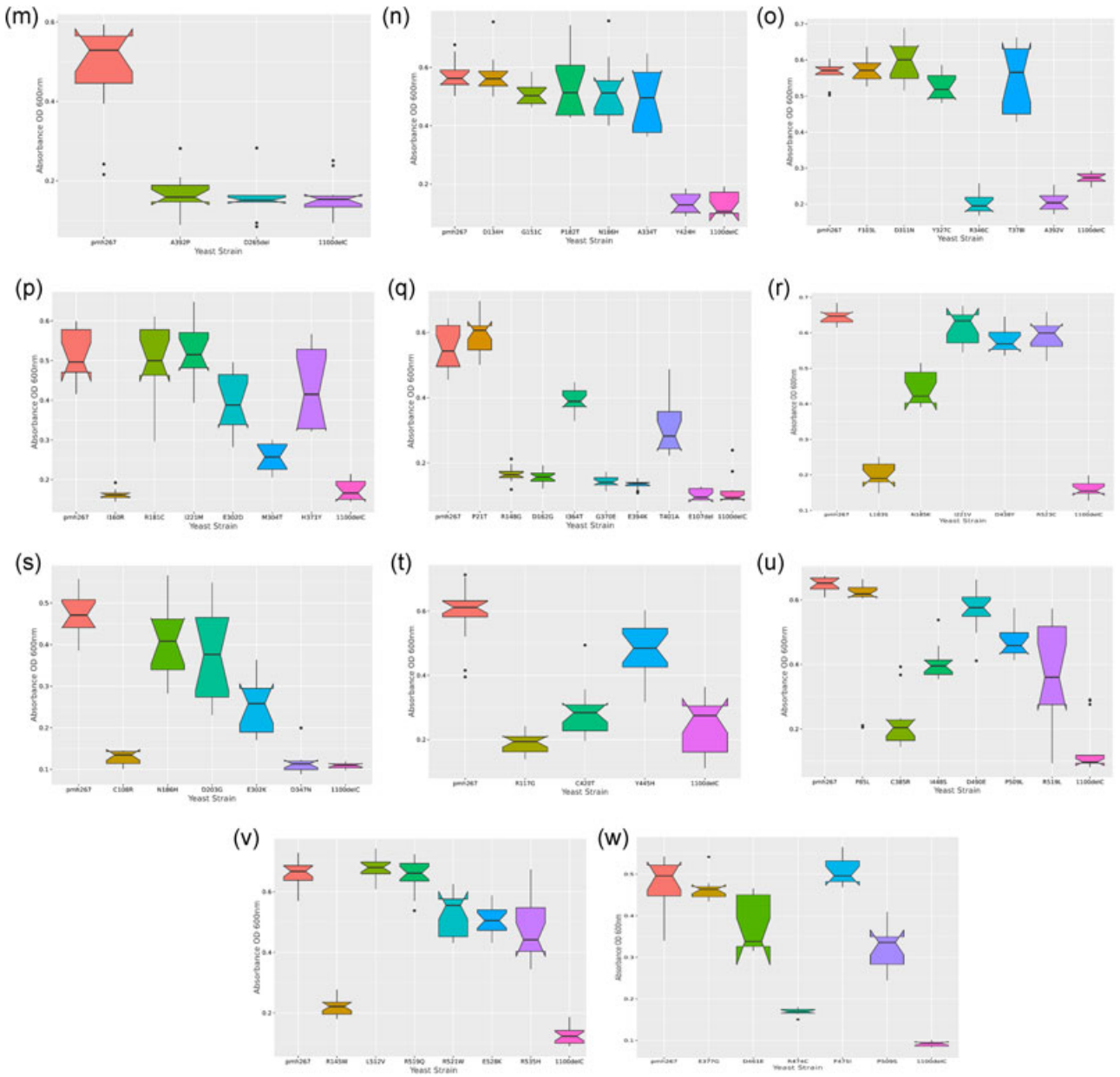

FIGURE 5 Continued

Presence of additional damaging alleles in other breast cancer predisposing genes has been excluded, since all *CHEK2* variant carriers have been tested using a 94-gene panel (data not shown).

4 | DISCUSSION

As increasing genetic data emerge from the extended use of multigene panels, it is evident that *CHEK2* variants represent a significant burden of the genetic information to be evaluated and communicated to breast cancer patients and their physicians. According to the data deriving from *CHEK2* truncating variants, female carriers face a ~25–30% lifetime risk for breast cancer diagnosis (Couch et al., 2017; Hauke et al., 2018; Slavin et al., 2017), while actual breast cancer risk associated with rare *CHEK2* missense variants, is challenging, due to limited epidemiological and functional data. As a first step, their functional characterization through an in vivo functional assay, such as the one presented here, will provide the basis for variant classification that will be further enriched with genetic and epidemiological information.

In this study, the largest to date yeast-based assay assessment of *CHEK2* unclassified variants, 27% (32/120) of them behaved as loss-of-function, with the majority being located at the kinase and the FHA domains of CHK2, as indicated by the structural model, while in silico predictions, to a great extent, were in agreement with the functional results.

TABLE 1 Categorization of *CHEK2* variants according to in silico tools and yeast functional assay (in vivo results)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.7C>T	p.(Arg3Trp)	-	*Damaging	C25	Probably damaging	1	2.124	Polymorphism (0.869)	3.34	101	0.0233	Benign
c.14C>T	p.(Ser5Leu)	-	*Damaging	C0	Benign	0.191	0.362	Polymorphism (0.999)	−0.66	145	0.0116	Benign
c.61C>A	p.(Pro21Thr)	SCD	Tolerated	C0	Benign	0.576	−1.206	Polymorphism (0.997)	N.A.	N.A.	N.A.	Benign
c.170C>T	p.(Ser57Phe)	SCD	Tolerated	C65	Probably damaging	0.998	4.061	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.190G>A	p.(Glu64Lys)	SCD	Tolerated	C15	Benign	1	2.612	Disease causing (0.807)	4.41	56	0.0698	Benign
c.202A>G	p.(Thr68Ala)	SCD	Tolerated	C0	Possibly damaging	0.987	2.063	Disease causing (0.863)	N.A.	N.A.	N.A.	Benign
c.239C>A	p.(Pro80His)	-	Tolerated	C0	Probably damaging	0.849	2.527	Disease causing (0.923)	N.A.	N.A.	N.A.	Benign
c.254C>T	p.(Pro85Leu)	-	Tolerated	C0	Possibly damaging	0.877	1.372	Polymorphism (0.824)	N.A.	N.A.	N.A.	Benign
c.277T>C	p.(Trp93Arg)	-	Damaging	C65	Probably damaging	1	3.39	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.307T>C	p.(Phe103Leu)	-	Tolerated	C15	Benign	1	3.348	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.322T>C	p.(Cys108Arg)	-	Damaging	C65	Probably damaging	1	3.825	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.349A>G	p.(Arg117Gly)	FHA	Damaging	C65	Probably damaging	1	1.49	Disease causing (0.999)	4.81	125	0.0116	Damaging
c.368A>G	p.(Tyr123Cys)	FHA	Tolerated	C65	Benign	1	3.892	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.400G>C	p.(Asp134His)	FHA	Tolerated	C0	Possibly damaging	0.52	1.392	Polymorphism (0.986)	4.86	81	0.0116	Benign
c.410G>A	p.(Arg137Gln)	FHA	Tolerated	C0	Benign	0.997	0.834	Polymorphism (0.556)	1.56	43	0.00116	Benign
c.433C>T	p.(Arg145Trp)	FHA	Damaging	C65	Probably damaging	0.999	1.516	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.434G>A	p.(Arg145Gln)	FHA	Tolerated	C35	Probably damaging	1	4.69	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.442A>G	p.(Arg148Gly)	FHA	Damaging	C25	Probably damaging	1	0.868	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.443G>C	p.(Arg148Thr)	FHA	Damaging	C0	Probably damaging	1	4.69	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.451G>T	p.(Gly151Cys)	FHA	Damaging	C0	Benign	0.999	2.736	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.470T>C	p.(Ile157Thr)	FHA	Tolerated	C25	Possibly damaging	1	3.51	Disease causing (0.998)	5.87	89	0.2442	Benign
c.475T>C	p.(Tyr159His)	FHA	Damaging	C65	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign

(Continues)

TABLE 1 (Continued)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.479T>G	p.(Ile160Arg)	FHA	Damaging	C65	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.479T>C	p.(Ile160Thr)	FHA	Damaging	C65	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.480A>G	p.(Ile160Met)	FHA	Damaging	C0	Probably damaging	0.982	−0.276	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.485A>G	p.(Asp162Gly)	FHA	Damaging	C65	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.497A>G	p.(Asn166Ser)	FHA	Damaging	C45	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.499G>A	p.(Gly167Arg)	FHA	Damaging	C65	Probably damaging	1	4.205	Disease causing (0.999)	5.87	125	0.0116	Damaging
c.514A>G	p.(Thr172Ala)	FHA	Tolerated	C0	Benign	0.79	1.207	Polymorphism (0.998)	N.A.	N.A.	N.A.	Benign
c.520C>T	p.(Leu174Phe)	FHA	Tolerated	C0	Probably damaging	0.984	0.65	Disease causing (0.936)	N.A.	N.A.	N.A.	Benign
c.522T>C	p.(Val175Ala)	FHA	Damaging	C25	Possibly damaging	0.949	−0.319	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.538C>T	p.(Arg180Cys)	-	Damaging	C25	Possibly damaging	1	2.202	Disease causing (0.999)	5.87	180	0.1279	Benign
c.539G>A	p.(Arg180His)	-	Damaging	C0	Benign	1	1.882	Disease causing (0.997)	N.A.	N.A.	N.A.	Intermediate
c.541C>T	p.(Arg181Cys)	-	Tolerated	C0	Benign	0.998	1.24	Disease causing (0.994)	3.76	180	0.0116	Benign
c.542G>A	p.(Arg181His)	-	Tolerated	C0	Benign	1	1.489	Polymorphism (0.628)	N.A.	N.A.	N.A.	Benign
c.544C>A	p.(Pro182Thr)	-	Tolerated	C0	Benign	0.998	2.627	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.548T>C	p.(Leu183Ser)	-	Damaging	C65	Probably damaging	0.988	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.549G>C	p.(Leu183Phe)	-	Damaging	C15	Probably damaging	0.995	1.263	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.555C>G	p.(Asn185Lys)	-	Damaging	C0	Probably damaging	1	1.197	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.556A>C	p.(Asn186His)	-	Damaging	C65	Probably damaging	1	3.51	Disease causing (0.999)	5.87	68	0.0116	Benign
c.565A>G	p.(Ile189Val)	-	Tolerated	C25	Probably damaging	1	3.51	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.608A>G	p.(Asp203Gly)	-	Damaging	C65	Probably damaging	1	3.688	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign

(Continues)

TABLE 1 (Continued)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.613A>T	p.(Thr205Ser)	-	Tolerated	C0	Benign	1	2.118	Disease causing (0.848)	N.A.	N.A.	N.A.	Benign
c.620A>T	p.(Asp207Val)	-	Tolerated	C65	Possibly damaging	1	3.688	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.661A>G	p.(Ile221Val)	-	Tolerated	C0	Benign	1	2.172	Disease causing (0.995)	5.51	29	0.0117	Benign
c.663C>G	p.(Ile221Met)	-	Tolerated	C0	Possibly damaging	1	2.974	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.664A>G	p.(Met222Val)	-	Tolerated	C0	Benign	1	1.396	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.688G>T	p.(Ala230Ser)	Kinase	Tolerated	C15	Benign	1	3.318	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.707T>C	p.(Leu236Pro)	Kinase	Damaging	C45	Probably damaging	1	2.785	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.715G>A	p.(Glu239Lys)	Kinase	Tolerated	C15	Probably damaging	1	3.376	Disease causing (0.999)	5.27	56	0.0233	Benign
c.715G>C	p.(Glu239Gln)	Kinase	Tolerated	C0	Probably damaging	1	3.376	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.727T>C	p.(Cys243Arg)	Kinase	Tolerated	C0	Possibly damaging	0.981	2.834	Disease causing (0.999)	5.27	180	0.0116	Benign
c.751A>T	p.(Ile251Phe)	Kinase	Tolerated	C15	Probably damaging	1	2.834	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.787G>C	p.(Glu263Gln)	Kinase	Tolerated	C0	Benign	0.857	3.274	Disease causing (0.963)	N.A.	N.A.	N.A.	Benign
c.842A>G	p.(Asn281Ser)	Kinase	Tolerated	C15	Benign	0.999	2.703	Disease causing (0.572)	N.A.	N.A.	N.A.	Benign
c.904G>A	p.(Glu302Lys)	Kinase	Damaging	C55	Probably damaging	1	2.602	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.906A>C	p.(Glu302Asp)	Kinase	Damaging	C35	Possibly damaging	1	0.824	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.911T>C	p.(Met304Thr)	Kinase	Tolerated	C55	Probably damaging	1	4.142	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.917G>C	p.(Gly306Ala)	Kinase	Tolerated	C55	Probably damaging	1	5.005	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.926T>C	p.(Leu309Pro)	Kinase	Damaging	C65	Probably damaging	1	4.142	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.931G>A	p.(Asp311Asn)	Kinase	Tolerated	C0	Benign	1	2.434	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.952C>T	p.(Arg318Cys)	Kinase	Tolerated	C15	Probably damaging	0.564	0.762	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.980A>G	p.(Tyr327Cys)	Kinase	Damaging	C65	Probably damaging	1	4.142	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1000G<A	p.(Ala334Thr)	Kinase	Damaging	C55	Probably damaging	1	5.005	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign

(Continues)

TABLE 1 (Continued)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.1012C>T	p.(Leu338Phe)	Kinase	Damaging	C15	Probably damaging	1	4.665	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1024G>A	p.(Gly342Ser)	Kinase	Damaging	C55	Probably damaging	1	4.665	Disease causing (0.999)	N.A.	N.A.	N.A.	benign
c.1036C>T	p.(Arg346Cys)	Kinase	Damaging	C65	Probably damaging	1	1.417	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1037G>A	p.(Arg346His)	Kinase	Damaging	C25	Probably damaging	1	4.73	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1039G>A	p.(Asp347Asn)	Kinase	Damaging	C15	Probably damaging	1	4.73	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1040A>C	p.(Asp347Ala)	Kinase	Damaging	C65	Probably damaging	1	2.48	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1054A>G	p.(Asn352Asp)	Kinase	Damaging	C15	Probably damaging	1	3.928	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1067C>T	p.(Ser356Leu)	Kinase	Tolerated	C15	Probably damaging	1	4.73	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1091T>C	p.(Ile364Thr)	Kinase	Damaging	C65	Probably damaging	1	3.928	Disease causing (1)	N.A.	N.A.	N.A.	Intermediate
c.1109G>A	p.(Gly370Glu)	Kinase	Damaging	C0	Probably damaging	1	3.517	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1111C>T	p.(His371Tyr)	Kinase	Damaging	C35	Benign	1	5.469	Disease causing (0.998)	N.A.	N.A.	N.A.	Benign
c.1118A>G	p.(Lys373Arg)	Kinase	Tolerated	C25	Benign	1	2.815	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1130A>G	p.(Glu377Gly)	Kinase	Damaging	C65	Possibly damaging	1	4.53	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1133C>T	p.(Thr378Ile)	Kinase	Tolerated	C15	Benign	1	5.469	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1141A>G	p.(Met381Val)	Kinase	Tolerated	C15	Possibly damaging	1	4.53	Disease causing (0.999)	5.89	21	0.0349	Benign
c.1153T>C	p.(Cys385Arg)	Kinase	Damaging	C65	Probably damaging	1	4.53	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1160C>G	p.(Thr387Ser)	Kinase	Damaging	C55	Benign	1	5.469	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1169A>C	p.(Tyr390Ser)	Kinase	Damaging	C65	Probably damaging	0.995	4.53	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1174G>C	p.(Ala392Pro)	Kinase	Damaging	C25	Probably damaging	1	5.392	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1175C>T	p.(Ala392Val)	Kinase	Damaging	C65	Probably damaging	1	5.392	Disease causing (0.999)	5.73	64	0.0116	Damaging

(Continues)

TABLE 1 (Continued)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.1180G>A	p.(Glu394Lys)	Kinase	Damaging	C55	Probably damaging	1	5.392	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1183G>C	p.(Val395Leu)	Kinase	Damaging	C25	Probably damaging	1	3.805	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1201A>G	p.(Thr401Ala)	Kinase	Tolerated	C0	Benign	0.857	1.972	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.1215C>A	p.(Asn405Lys)	Kinase	Tolerated	C0	Benign	0.842	0.227	Polymorphism (0.992)	N.A.	N.A.	N.A.	Benign
c.1216C>T	p.(Arg406Cys)	Kinase	Tolerated	C15	Possibly damaging	0.838	1.772	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1217G>A	p.(Arg406His)	Kinase	Damaging	C0	Probably damaging	0.705	1.224	Disease causing (0.983)	3.53	29	0.0116	Benign
c.1259G>A	p.(Cys420Thr)	Kinase	Damaging	C65	Probably damaging	1	5.392	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1270T>C	p.(Tyr424His)	Kinase	Tolerated	C65	Possibly damaging	1	4.282	Disease causing (0.999)	5.81	83	0.0349	Damaging
c.1312G>T	p.(Asp438Tyr)	Kinase	Damaging	C25	Probably damaging	1	3.555	Disease causing (0.999)	5.81	160	0.0349	Benign
c.1313A>G	p.(Asp438Gly)	Kinase	Damaging	C15	Possibly damaging	1	4.282	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1333T>C	p. (Tyr445His)	Kinase	Damaging	C25	Probably damaging	1	2.793	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.1336A>G	p.(Asn446Asp)	Kinase	Tolerated	C0	Benign	1	1.799	Polymorphism (0.902)	N.A.	N.A.	N.A.	Benign
c.1343T>G	p.(Ile448Ser)	Kinase	Tolerated	C65	Benign	1	4.323	Disease causing (0.999)	5.91	1.42	0.0116	Intermediate
c.1383C>G	p.(Asp461Glu)	Kinase	Damaging	C35	Benign	0.997	0.155	Disease causing (0.999)	N.A.	N.A.	N.A.	Intermediate
c.1420C>T	p.(Arg474Cys)	Kinase	Damaging	C65	Probably damaging	0.87	1.865	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1423T>A	p.(Phe475Ile)	Kinase	Tolerated	C0	Benign	0.754	0.152	Polymorphism (0.993)	0.89	21	0.0213	Benign
c.1427C>T	p.(Thr476Met)	Kinase	Damaging	C15	Probably damaging	0.805	1.907	Disease causing (0.999)	4.43	81	0.0639	Intermediate
c.1448A>G	p.(His483Arg)	Kinase	Damaging	C25	Probably damaging	1	3.723	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.1450C>A	p.(Pro484Thr)	Kinase	Damaging	C35	Possibly damaging	1	4.485	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1451C>T	p.(Pro484Leu)	Kinase	Damaging	C65	Probably damaging	0.998	4.485	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1470C>A	p.(Asp490Glu)	-	Tolerated	C0	Benign	0.997	0.663	Disease causing (0.959)	N.A.	N.A.	N.A.	Benign
c.1489G>A	p.(Asp497Asn)	-	Tolerated	C0	Benign	0	−0.118	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1501G>A	p.(Glu501Lys)	-	Tolerated	C0	Benign	0.453	0.632	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign

(Continues)

TABLE 1 (Continued)

Variants												
cDNA (NM_007194.3)	Protein (NP_009125.1)	Domain	SIFT/ PROVEAN	Align GVGD[a]	PolyPhen-2	PhastCons[b]	PhyloP[c]	MutationTaster	GERP[d]	Grantham[e]	MAF (EA)	*In vivo* results -Characterization
c.1510G>C	p.(Glu504Gln)	-	Tolerated	C0	Benign	0	0.129	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1513T>A	p.(Ser505Thr)	-	Tolerated	C0	Benign	0.001	0.325	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1525C>T	p.(Pro509Ser)	-	Tolerated	C0	Benign	0	−0.049	Polymorphism (0.999)	N.A.	N.A.	N.A.	Intermediate
c.1526C>T	p.(Pro509Leu)	-	Tolerated	C0	Benign	0.004	1.338	Polymorphism (0.999)	N.A.	N.A.	N.A.	Intermediate
c.1534C>G	p.(Leu512Val)	-	Tolerated	C0	Benign	0.002	0.603	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1556G>A	p.(Arg519Gln)	NLS	Tolerated	C0	Benign	0.976	−0.295	Disease causing (0.620)	N.A.	N.A.	N.A.	Intermediate
c.1556G>T	p.(Arg519Leu)	NLS	Damaging	C25	Possibly damaging	0.976	−0.295	Disease causing (0.921)	N.A.	N.A.	N.A.	Intermediate
c.1561C>T	p.(Arg521Trp)	NLS	Damaging	C65	Probably damaging	1	2.918	Disease causing (0.999)	N.A.	N.A.	N.A.	Benign
c.1567C>T	p.(Arg523Cys)	-	Tolerated	C15	Possibly damaging	0.159	1.574	Polymorphism (0.999)	3.73	180	0.0218	Benign
c.1582G>A	p.(Glu528Lys)	-	Tolerated	C15	Possibly damaging	0.736	2.918	Disease causing (0.978)	4.76	56	0.0218	Benign
c.1591G>C	p.(Glu531Gln)	-	Tolerated	C0	Benign	0	0.298	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1597A>G	p.(Thr533Ala)	-	Tolerated	C0	Benign	0.017	−0.369	Polymorphism (0.999)	N.A.	N.A.	N.A.	Benign
c.1604G>A	p.(Arg535His)	-	Tolerated	C0	Probably damaging	0.189	0.725	Polymorphism (0.999)	N.A.	N.A.	N.A.	Intermediate
c.320_592del6160	p.(Glu107_Lys197del)	-	Deleterious	-	-	-	-	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging
c.793_846del7566	p.(Asp265_His282del)	Kinase	Deleterious	-	-	-	-	Disease causing (0.999)	N.A.	N.A.	N.A.	Damaging

Note. FHA: forkhead-associated; MAF: minor allele frequency; NLS: nuclear localization signal; SCD: SQ/TQ cluster domain.*CHEK2* missense variants detected in Greek breast cancer families have been underlined.

[a]Align GVGD combines the biophysical characteristics of amino acids and multiple protein sequence alignments to predict the effect of missense substitutions. C65 is most likely to interfere with function. Predictions were derived from the website's human *CHEK2* alignment to the zebrafish depth. C0-C15 are considered as benign, C25-C35 as intermediate, and C35-C65 as deleterious.

[b]PhastCons estimates the probability that each nucleotide belongs to a conserved element and ranges between 0 and 1.

[c]PhyloP is the -log(*p*-value) under a null hypothesis of neutral evolution. Negative score indicates faster than expected evolution, while positive sign implies conservation.

[d]GERP is a software that uses algorithms to quantify the level of evolutionary constraint acting on each site. It ranges from −12.36 to 6.18, with 6.18 being the most conserved.

[e]Grantham is the prediction of the effect of amino acids substitutions based on chemical properties. The characterization could be conservative (0–50), moderately conservative (51–100), moderately radical (101–150), or radical (≥151).

TABLE 2 Structural insights on the effect of tested missense variants based on inspection of the CHK2 crystal structure with Protein Data Bank code 3I6U

Variants	Possible effects on structure and function
p.(Trp93Arg)	Buried hydrophobic residue; change to a charged residue will have strong consequences to thermodynamic stability and folding
p.(Cys108Arg)	Buried hydrophobic residue; change to a charged residue will have strong consequences to thermodynamic stability and folding
p.(Arg145Trp)	Semiburied Arg residue is changed. Stabilizing interactions with Trp114 and Phe147 will be perturbed, leading to reduced thermodynamic stability or folding efficiency (Figure 3a)
p.(Arg148Gly)	Semiburied Arg residue is changed. Stabilizing interactions with Trp93 and Tyr156 will be perturbed, leading to reduced thermodynamic stability or folding efficiency (Figure 3b)
p.(Ile160Arg), p.(Ile160Thr), p.(Ile160Met)	Buried hydrophobic residue in wild-type protein. Change to hydrophilic Arg or Thr will affect stability/folding, while change to Met may be less detrimental
p.(Leu183Phe), p.(Leu183Ser)	Wild-type residue is buried between two β-sheets. Change may destabilize protein or folding
p.(Arg346Cys)	Wild-type residue is exposed. Change to Cys may promote disulfide bond formation and aberrant dimerization
p.(Thr387Ser)	Not visible in crystal structure. Variant affects the conservative residue and may have limited effects on structure/function
p.(Ala392Pro)	Nonconservative change, it may disrupt local structure due to helical segment destabilization by Pro. May also affect folding
p.(Tyr424His)	Mostly buried, Tyr hydroxyl group possibly makes hydrogen bond with backbone carbonyl; change may disrupt local structure
p.(Arg474Cys)	Wild-type residue is mostly buried and stabilized by a salt bridge with Glu394 and is important for the configuration of 367–374 loop and 391–396 region. Change will destabilize local structure (Figure 3d)
p.(His483Arg)	Wild-type residue is partially buried and makes hydrogen bonding interactions with Asp461; change will disrupt interaction between two adjacent helices and possibly generate repulsion with Lys465 (Figure 3e)
p.(Ile157Thr)	Residue is hydrophobic and buried. Change to a hydrophilic residue will destabilize structure and possibly folding. Plays a central role in the FHA-kinase domain interface, mutation may affect dimerization and autophosphorylation
p.(Tyr159His)	Residue is buried and makes π-stacking interactions with Arg180, and hydrogen bonds with Lys245. Change may have moderate effect on local structure and stability but may also affect dimerization since it is at the FHA-KD interface (Figure 3c)

Note. FHA: forkhead-associated.

Although a small number of the variants tested by our assay has been previously tested by a functional assay, we evaluated our results with published data from functional assays, which were discordant, to some extent. The discrepancies observed can be attributed to variable conditions used during experimentation, that is, the MMS concentration used, along with the time of measurement after DNA damage, are significant factors. More specifically, the MMS concentration of 0.014% used in a previous study (Roeb et al.), proved to be toxic in our assay, while the 48-hr starting time-point for their measurement, was insufficient for us, since there were no differences observed between the tested variants and controls at this time, probably due to MMS degradation and media evaporation. Moreover, as it was evident after a small number of experiments in our hands, a 10-fold dilution of yeast cultures in our assay provided clearer results.

Among the discrepancies, the well-studied, but controversial, *CHEK2* p.Ile157Thr variant was included. It was characterized as benign by us and Lee et al, but as damaging by Roeb et al. In silico predictions performed were contradictive. In these, MAF was found relatively high, whereas the structural prediction model revealed a possible impairment in autophosphorylation after dimerization. When tested in a human colorectal carcinoma cell line by immunoprecipitation, immunoblotting, and kinase assays, the variant presented similar behavior with the wild-type protein (Wu et al., 2001), while inactivation of Cdc25C, which is crucial for G2 arrest after DNA damage, was feasible, but binding with Cdc25A, p53, and BRCA1, was impaired (Falck, Lukas, et al., 2001; Falck, Mailand, et al., 2001; Li et al., 2002). Consequently, the produced protein was as stable as the wild-type CHK2, whereas, in the same study, it was shown that protein expression levels in breast tumors were not elevated, advocating for the benign characterization of this variant (Kilpivaara et al., 2004).

Moreover, various studies showed co-occurrence of p.(Ile157Thr) variant with pathogenic alleles in *CHEK2*, *BRCA1* and *BRCA2* genes. In a large meta-analysis, assessing data from 18 case-control studies, *CHEK2* p.(Ile157Thr) was considered to pose an increased risk of breast cancer with an odds ratio of 1.58 (Han, Guo, & Liu, 2013). Therefore, our yeast-based assay result, in conjunction with the aforementioned evidence, supports the categorization of p.(Ile157Thr) as a non-damaging, possibly low-risk variant.

TABLE 3 Testing for concordance of results of yeast-based and cell line-based functional assays for common *CHEK2* variants tested

Variants		*CHEK2* functional assay studies				
cDNA (NM_007194.3)	Protein (NP_009125.1)	Present study	Roeb et al. (2012)[b]	Tischkowitz et al. (2008)[b]	Bell et al. (2007)[b]	Lee et al. (2001)[b]
c. 7C>T	p.(Arg3Trp)[a]	Benign	Intermediate			Benign
c.190G>A	p.(Glu64Lys)	Benign	Damaging			
c.254C>T	p.(Pro85Leu)[a]	Benign	Benign		Intermediate	
c.349A>G	p.(Arg117Gly)[a]	Damaging	Damaging			
c.410G>A	p.(Arg137Gln)[a]	Benign	Benign		Benign	
c.433C>T	p.(Arg145Trp)[a]	Damaging	Damaging			Damaging
c.470T>C	p.(Ile157Thr)[a]	Benign	Damaging			Benign
c.480A>G	p.(Ile160Met)	Benign	Intermediate			
c.499G>A	p.(Gly167Arg)[a]	Damaging	Damaging			
c.538C>T	p.(Arg180Cys)	Benign	Intermediate			
c.541C>T	p.(Arg181Cys)	Benign	Intermediate			
c.565A>G	p.(Ile189Val)	Benign	Damaging			
c.715G>A	p.(Glu239Lys)	Benign	Intermediate			
c.917G>C	p.(Gly306Ala)	Benign	Damaging			
c.1040A>C	p.(Asp347Ala)[a]	Damaging	Damaging			
c.1270T>C	p.(Tyr424His)[a]	Damaging	Damaging	Benign		
c.1312G>T	p.(Asp438Tyr)	Benign			Intermediate	
c.1427C>T	p.(Thr476Met)	Intermediate	Damaging			

[a]The characterization of these *CHEK2* missense variants by our yeast-based functional assay is in concordance with the result of at least one other functional assay study.

[b]The overall tested missense variants in other functional studies were 26 in the study by Roeb et al., two in the study by Tischkowitz et al. (2008), four in the study by Bell et al. (2007), and three in the study by Lee et al. (2001).

In this work, an unexpected finding was the classification of p.(Thr68Ala) as a benign variant. In the case of DNA double-strand breaks, ATM protein kinase phosphorylates CHK2 at Thr68, along with other residues in the SQ/TQ domain, leading to CHK2 dimerization (Ahn, Urist, & Prives, 2004).

In *Saccharomyces cerevisiae*, activation of RAD53 protein in response to DNA damage depends on phosphorylation by MEC1, and to a lower extent by TEL1, the closest ATM homologue in yeast (S. J. Lee, Schwartz, Duong, & Stern, 2003; Sanchez et al., 1996). However, previous studies have shown that RAD53 overexpression in bacteria results in its hyperphosphorylation, with no other protein being required for this (Gilbert, Green, & Lowndes, 2001; S. J. Lee et al., 2003). Thus, characterization of p.(Thr68Ala) as non-damaging can be possibly attributed to CHK2 overexpression in yeast.

Another important finding of our study is the characterization of p.(Thr387Ser) as damaging, which, along with Thr383, are the CHK2 autophosphorylation residues, providing evidence that, after initial CHK2 phosphorylation, downstream events leading to complete CHK2 activation in yeast, require these sites the same way as in humans (C. H. Lee & Chung, 2001).

A number of tested variants were classified as benign by our yeast functional assay and were in disagreement with in silico tools. In order for this discrepancy to be solved, additional experimental and clinical data are necessary to produce a definite classification of *CHEK2* missense variants. In addition, further investigation is needed on variants characterized as intermediate by this functional assay, to clarify their pathogenicity.

The major limitation of this assay is the fact that it is yeast-based, thus the results cannot be blindly extrapolated to humans. Hence, we should be cautious when addressing clinical significance. The results from the yeast functional assay are to be considered as indications, complementary to additional clinical and experimental data and cannot stand alone when attempting to predict *CHEK2* variant pathogenicity, when assessing the repairability of CHK2. Therefore, a combination of information from functional assays conducted in human cell lines, as well as data from epidemiological and genetic studies, and importantly, segregation analysis results, would provide more accurate classifications. Unfortunately, detailed segregation analyses were not feasible in the context of this study. Moreover, the interference of the tested variants in the canonical splicing process cannot be assessed by our yeast-based functional assay. However, in the case where the actual effect on splicing is known, the predicted constructs can be evaluated by the assay.

As multiple *CHEK2* variants of unknown clinical significance emerge every day when performing genetic testing analyses in patients with cancer, a rapid variant assessment is of great importance. Therefore, the in vivo functional assay developed and used herein provides essential, fast, and low-cost evaluation for

the largest series of tested *CHEK2* variants (including missense variants and in-frame insertions/deletions) to date, thus providing valuable information that can be ultimately implemented in clinical practice.

ACKNOWLEDGMENTS

The authors thank the patients and their families for their participation in this study. They also thank ENIGMA members, Amanda Toland, Soo-Hwang Teo, and Conxi Lazaro, for providing information about *CHEK2* variants.

FUNDING INFORMATION

This work was funded by the Greek State Scholarships Foundation (IKY), the A. G. Leventis Foundation, and the research program ARISTEIA. This research was cofinanced by the European Union (European Social Fund, ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) Research Funding Program of the General Secretariat for Research & Technology (ARISTEIA 39, P. BROCA), which invests in knowledge society through the ESF. The authors acknowledge support of this work by the project "Target Identification and Development of Novel Approaches for Health and Environmental Applications" (MIS 5002514), which is implemented under the Action for the Strategic Development on the Research and Technological Sectors, funded by the Operational Programme "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014–2020) and cofinanced by Greece and the European Union (European Regional Development Fund).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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SUPPORTING INFORMATION

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How to cite this article: Delimitsou A, Fostira F, Kalfakakou D, et al. Functional characterization of *CHEK2* variants in a *Saccharomyces cerevisiae* system. *Human Mutation*. 2019;1–18. https://doi.org/10.1002/humu.23728