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Functional analysis of genetic variants in PALB2 and CHEK2: linking functional impact with cancer risk

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CHAPTER 2

Functional characterization of *PALB2* variants of uncertain significance: towards cancer risk and therapy response prediction

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ABSTRACT

In recent years it has become clear that pathogenic variants in *PALB2* are associated with a high risk for breast, ovarian and pancreatic cancer. However, the clinical relevance of variants of uncertain significance (VUS) in *PALB2*, which are increasingly identified through clinical genetic testing, is unclear. Here we review recent advances in the functional characterization of VUS in *PALB2*. A combination of assays has been used to assess the impact of *PALB2* VUS on its function in DNA repair by homologous recombination, cell cycle regulation and the control of cellular levels of reactive oxygen species (ROS). We discuss the outcome of this comprehensive analysis of *PALB2* VUS, which showed that VUS in PALB2's Coiled-Coil (CC) domain can impair the interaction with BRCA1, whereas VUS in its WD40 domain affect PALB2 protein stability. Accordingly, the CC and WD40 domains of PALB2 represent hotspots for variants that impair PALB2 protein function. We also provide a future perspective on the high-throughput analysis of VUS in *PALB2*, as well as the functional characterization of variants that affect *PALB2* RNA splicing. Finally, we discuss how results from these functional assays can be valuable for predicting cancer risk and responsiveness to cancer therapy, such as treatment with PARP inhibitor- or platinum-based chemotherapy.

KEYWORDS

Breast Cancer; Variant of Uncertain Significance (VUS); PALB2; DNA Repair Homologous Recombination (HR); PARP inhibitor

PALB2 is essential for DSB repair by homologous recombination

The integrity of our genome is relentlessly challenged by exogenous and endogenous insults that can induce DNA damage. To respond to such genotoxic threats, cells have evolved a number of DNA damage signalling and repair mechanisms, jointly known as the DNA damage response (DDR). The DDR is able to handle a myriad of DNA damages of which DNA double strand breaks (DSBs) are considered among the most deleterious to the cell. Human cells possess at least five pathways for DSB repair: canonical nonhomologous end joining (c-NHEJ), alternative nonhomologous end-joining (a-NHEJ), single-strand annealing (SSA), break-induced replication (BIR), and homologous recombination (HR) (1,2). c-NHEJ is the predominant DSB repair pathway in human cells and complete loss-of-function (LOF) is likely to drive cell death due to an unreasonably high DSB burden (3). In case c-NHEJ fails or is inappropriate, HR is probably the most frequently used alternative pathway for DSB repair. However, while c-NHEJ is active throughout the whole cell cycle, HR is restricted to late S/G2 phase as it relies on the presence of an undamaged sister chromatid to act as a template for error free repair (4). During HR, BRCA1 inhibits 53BP1 from interacting with the chromatin near the broken DNA ends (2,5). This permits extensive end-resection of the break by endo- and exonucleases such as MRE11, CtIP, DNA2, and EXO1, yielding 3′-single-stranded (ss) DNA overhangs that counter Ku loading and further promote DSB repair by HR (6). Following resection, the 3′-ssDNA tails become coated by the RPA heterotrimer (7). Subsequently, BRCA1, PALB2 and BRCA2 sequentially accumulate on the processed ssDNA to promote error-free repair of DSBs.

PALB2 is crucial herein as it mediates PALB2-BRCA1/2-RAD51 complex formation. That is, PALB2's N-terminal Coiled-Coil (CC) domain is required for interaction with BRCA1, whereas its C-terminal WD40 domain mediates the interaction with BRCA2 (Fig. 1) (8-12). BRCA2 possesses eight highly conserved BRC repeats and a carboxy-terminal region that have been shown to bind RAD51 (13-15). This interaction allows BRCA2 to promote HR by facilitating the replacement of RPA with the RAD51 recombinase and by stabilizing the ensuing RAD51-ssDNA filaments through blockage of ATP hydrolysis (16). Additionally, through its WD40 domain, PALB2 also interacts with the C-terminal PALB2-Interacting Domain (PID) of the RNF168 ubiquitin E3 ligase. RNF168 contains a ubiquitin-interacting motif (UIM) that allows binding of RNF168-bound PALB2 to ubiquitylated chromatin at DSBs, thereby facilitating RAD51 filament formation and HR (17). Alternatively, more recent studies suggested that RNF168 may facilitate PALB2-mediated RAD51 loading independently of BRCA1, by showing that abrogation of RNF168 activity in BRCA1-compromised cells dramatically elevated genome instability rates (18,19). Thus, it is apparent that RAD51 loading during HR, regardless of its dependency on BRCA1, RNF168, or both, requires the action of PALB2.

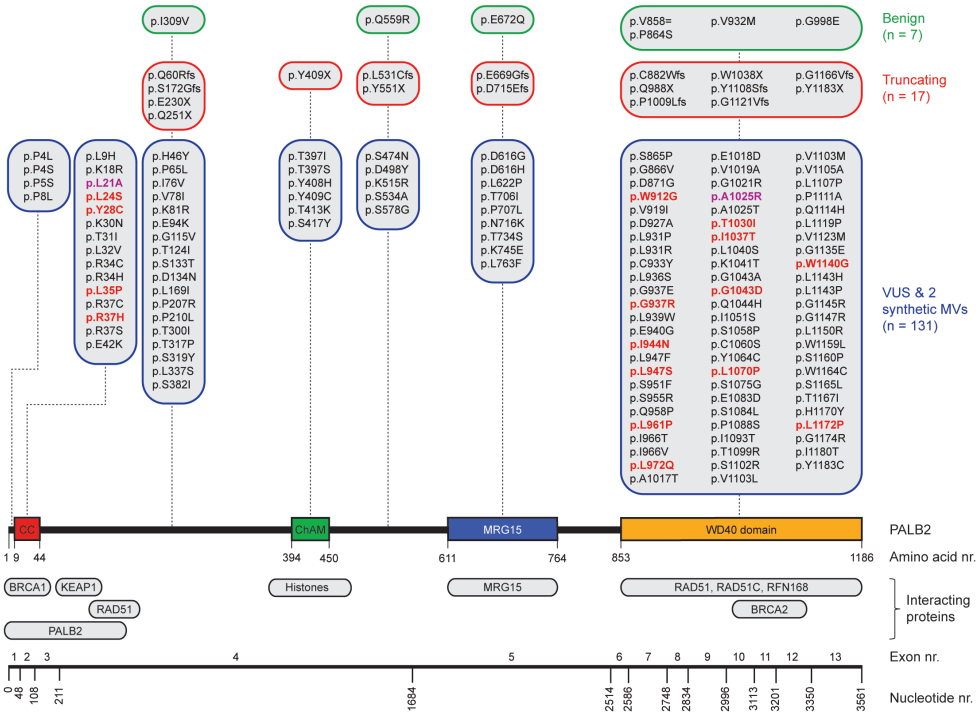

Figure 1. Schematic representation of *PALB2* variants, functional domains, interacting proteins and exons. The nucleotide numbers refer to the last nucleotide of each exon in *PALB2* cDNA (NM_024675.3). The amino acid numbers are shown to specify the evolutionarily conserved functional domains of PALB2; Coiled-coil (CC) (10-12,107), Chromatin-Association Motif (ChAM) (108), MORF-Related Gene on chromosome 15 (MRG15) binding domain (109) and WD40 domain (9,107). PALB2-interacting proteins are depicted underneath their respective PALB2 interacting domain/regions. All *PALB2* genetic variants from five functional studies (39-43) are shown and categorized per (functional) domain as benign (green framed sections), truncating (red framed sections), or VUS and synthetic missense variants (MVs) (blue framed sections) based on ClinVar. All functionally damaging *PALB2* VUS with an HR efficiency < 50% compared to wild type *PALB2* in at least one functional assay are highlighted in red. The two damaging synthetic MVs are highlighted in purple.

Genetic variants in *PALB2* and their association with cancer

Recent analysis of a metastatic pan-cancer cohort of 3504 patients, employing a strategy that relies on the presence of specific mutational footprints which are characteristic of a deficiency in HR (20,21), revealed that mutational inactivation of the *BRCA1*, *BRCA2* and *PALB2* genes, was the most common genetic cause of the observed HR signatures (22), indicative of their important role in tumor suppression. Indeed, for *BRCA1* and *BRCA2*, monoallelic LOF variants present in the germline can result in a nearly tenfold increased lifetime risk of developing breast cancer (23,24), whereas bi-allelic LOF variants cause Fanconi anemia (FA) (25,26). The

PALB2 gene, which is located on chromosome 16p12.2, comprises 13 exons and encodes a protein of 1186-amino acids (Fig. 1), was identified in 2006 as an important BRCA2-interacting protein (9,27). As it has now been established that LOF variants in *PALB2* convey a similarly high risk for breast cancer as *BRCA2* LOF variants (23,24,28), *PALB2* has become widely included in breast cancer clinical genetics practice. Consequently, a large number of people have already undergone genetic testing of *PALB2* to identify variants that may increase the risk of breast cancer susceptibility. Meanwhile, truncating *PALB2* variants have also been shown to be associated with an increased risk of familial ovarian and pancreatic cancer (29-33).

In contrast to truncating variants in *PALB2*, which are known to be deleterious to protein function, the impact of most missense variants is often unclear. Generally, assessment of pathogenicity of such variants of uncertain significance (VUS) would rely mostly on in silico analysis, co-segregation of the variant with cancer, co-occurrence with pathogenic *PALB2* variants, and family history of cancer. However, for the majority of VUS, this information is not available and hence the associated cancer risk is unknown. To extend the utility of *PALB2* genetic test results, additional methods for interpreting VUS are therefore urgently required. Accordingly, recent independent studies have developed functional assays to determine the functional impact of a large number of *PALB2* VUS (Fig. 1). Here we review this comprehensive analysis, which highlights the CC and WD40 domains of PALB2 as hotspots for variants that impair its function in HR and cell cycle checkpoint regulation. Finally, we also highlight the value of this functional analysis in predicting the associated cancer risk and therapy response for VUS in *PALB2*.

A comprehensive functional analysis of VUS in *PALB2*

Assays using HR as a read-out have emerged as the standard for the functional characterisation of VUS in *BRCA1* and *BRCA2* (34-38). More recently, VUS in *PALB2* have also been characterised in a similar manner (Fig. 1 and Fig. 2) (39-43). To identify variants that impact HR, the well described DR-GFP reporter, as well as the more recently introduced CRISPR-LMNA HR assay were used (44,45). These assays rely on HR-mediated repair of a non-functional GFP gene and HR-mediated integration of a fluorescence marker at the *LMNA* locus, respectively (Fig. 2). Furthermore, PALB2 function was assessed by exposing cells that express a *PALB2* variant to PARPi or cisplatin (Fig. 2). Catalytic inhibition of PARP1 “traps” PARP1 molecules on endogenous ssDNA breaks, resulting in replication fork collapse and DSB formation (46). Cisplatin on the other hand, induces ~90% intra-strand cross-links and ~5% inter-strand cross-links (ICLs), the latter of which are converted into DSBs and predominantly repaired through the FA pathway (47). Both PARPi- and ICL-induced DSBs are repaired by HR. Consequently, in the absence of HR (e.g. due to PALB2 LOF), PARP-trapping

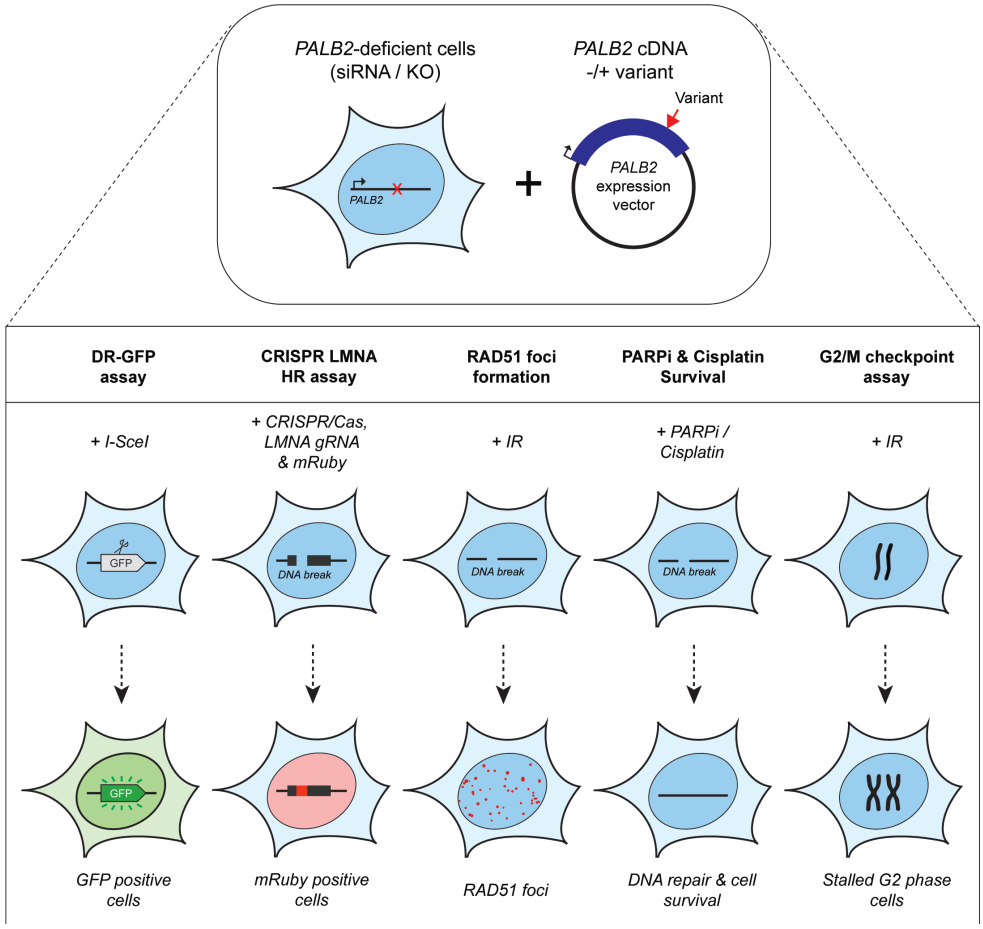

Figure 2. Overview of the functional assays used for the functional characterization of *PALB2* genetic variants. Either *Palb2* KO mouse cells or *PALB2* siRNA-depleted human cells were complemented by expressing human *PALB2* (siRNA-resistant) cDNA, without or with a variant. *PALB2* deficiency is indicated with a red cross, whereas a red arrow marks the position of a variant in the *PALB2* cDNA. Complementation was either by transient (B400 mouse cells or human cell lines) or stable expression (mES cells) (top section). *PALB2* complemented cells were subjected to multiple cell-based functional assays (bottom section). The functional assays determine in a quantitative manner: **1**) homology-directed repair of an I-*Sce*I–induced DSB in DR-GFP, which results in the restoration of a functional *GFP* gene whose expression can be monitored by fluorescence-activated cell sorting (FACS), **2**) HR-mediated integration of mRuby into the *LMNA* A/C locus (LMNA) at a break site induced by CRISPR/Cas9, **3**) the formation of IR-induced RAD51 foci, which is PALB2 dependent and provides a measurement for the HR efficiency, **4**) sensitivity to PARPi or cisplatin treatment, which leads to cell killing when HR is impaired, **5**) G2/M checkpoint maintenance after extensive DNA damage, which is dependent on PALB2-mediated HR. Deficiency in PALB2 results in progression into M-phase. Consequently, the mitotic fraction represents a measure for the functional impact of *PALB2* variants.

or ICL induction leads to persistent accumulation of DSBs. Such extensive DNA damage often results in cell cycle arrest and apoptosis, and thus, reduced proliferation and cell survival. PALB2 LOF is therefore synthetic lethal with PARPi or cisplatin treatment (48-50). Furthermore, PALB2 is also required for the repair of ionizing radiation-induced DSBs. This phenotype was used as a readout for the functional characterization of several *PALB2* variants, revealing that the expression of two variants, p.L939W and p.L1143P, impaired PALB2 functionally (41). Lastly, since PALB2 interacts with BRCA1 and BRCA2 to load RAD51 at sites of DSBs, co-immunoprecipitation, recruitment to micro irradiation-induced DNA damage, and DNA-damage-induced RAD51 foci formation were among the additional functional readouts to study the impact of PALB2 variants on HR (Fig. 2) (39-43). A complete overview of all functional assays that were performed by the three recent studies is provided in Table 1. With the above described functional assays, these studies analysed a total of 155 different *PALB2* variants (Table 2), comprising 129 VUS, 7 benign variants (as classified by ClinVar) (51), 2 synthetic missense variants with known LOF (11,52) and 17 truncating variants (Fig. 1). Sixteen VUS were identified as strongly damaging in at least one assay (i.e., >50% reduced activity compared to WT), all of which were located in the CC or WD40 domain of PALB2 (Fig. 1), highlighting the importance of these domains for PALB2's role HR. In the following sections, we review the different strategies and outcomes of these studies in more depth.

Functional characterization of VUS in *PALB2* using HR as a read-out

The largest set of *PALB2* variants, i.e., 84 patient-derived *PALB2* missense variants, was analysed by Wiltshire and colleagues (Table 2) (43). Several truncating variants (p.Q251X, p.Y551X p.D715Efs, p.Y1108Sfs, p.G1121Vfs, p.G1166Vfs and p.Y1183X) and benign missense variants as classified by ClinVar (p.I309V, p.Q559R, p.E672Q, p.P864S, p.V932M and p.G998E) were analysed to validate their functional impact. The assays were mostly performed in *Palb2*-deficient B400 mouse mammary tumour cells with a stably integrated DR-GFP reporter to measure HR. *PALB2* cDNA, with or without a variant, was transiently (over-) expressed in these cells and subsequently the effect on HR was determined. While benign variants had only a moderate or no impact on HR (<12% reduction in HR when compared to WT *PALB2*), all truncating variants strongly impacted HR (>52% reduction in HR when compared to WT *PALB2*). Moreover, four *PALB2* missense variants (p.L24S, p.L35P, p.I944N and p. L1070P) were identified that strongly disrupted HR (>65% reduction in HR compared to WT *PALB2*). To corroborate their findings, a CRISPR-*LMNA* HR assay (45) was performed in U2OS cells with endogenous *PALB2* depletion by siRNA treatment, followed by transient expression of siRNA-resistant *PALB2* cDNA with or without variant. Consistently, the same four variants disrupted HR-mediated mRuby integration into the *LMNA* locus. In this assay, the

variants exhibited a >90% reduction in HR compared to cells that were complemented with WT *PALB2* cDNA.

Table 1. Complete list of functional assays used in three independent studies.

Study	Functional assay	Nr. of variants tested (patient derived)
Boonen et al. 2019 (70 variants)	DR-GFP reporter	Complete set
	PARPi sensitivity (proliferation)	Complete set
	PALB2 expression blots	Complete set
	PARPi sensitivity (clonogenic)	8
	Cisplatin sensitivity (proliferation)	18
	RAD51 foci number after IR	5
	RAD51 foci intensity after IR	2
	CRISPR-LMNA HR	5
	G2>M checkpoint	19
	Micro-irradiation recruitment	3
	Co-immunoprecipitation	3
Rodrigue et al. 2019 (47 variants)	PARPi sensitivity proliferation assay	Complete set
	PALB2 expression blots	Complete set
	RAD51 foci number after IR	18
	RAD51 foci intensity after IR	8
	CRISPR-LMNA HR assay	18
	Micro-irradiation recruitment assay	18
	PALB2 cellular localization	18
	Mammalian two-hybrid assay (BRCA1) (1-319)	22
	Mammalian two-hybrid assay (BRCA2) (859-1186)	25
Wiltshire et al. 2019 (91 variants)	DR-GFP reporter	Complete set
	PARPi sensitivity (proliferation)	5
	PALB2 expression blots	6
	Cisplatin sensitivity (proliferation)	5
	RAD51 foci number after IR	4
	CRISPR-LMNA HR assay	4
	Micro-irradiation recruitment	4
	PALB2 cellular localization	4
	Mammalian two-hybrid assay (BRCA1) (1-319)	3
	Mammalian two-hybrid assay (BRCA2) (859-1186)	3
	Cyclohexamide chase / Stability	5
	Co-immunoprecipitation	6

Rodrigue and colleagues first tested their set of 41 *PALB2* VUS using PARPi sensitivity assays (Table 2) (42). Their assay was set up in HeLa cells in which endogenous *PALB2* was depleted by siRNA treatment. Following transient expression of siRNA-resistant YFP-*PALB2* cDNA, with or without a variant, cells were assayed for PARPi sensitivity. Although no truncating variants were assayed, several benign *PALB2* variants were included (i.e. p.P864S, p.V932M and p.G998E). As expected, expression of the benign variants rendered cells PARPi resistant, which was comparable to that observed after WT *PALB2* expression (42). The threshold for impaired PALB2 function was set based on the PARPi sensitivity observed for cells expressing p.L35P (~50% survival), which was previously reported to be damaging (40). The expression of two *PALB2* variants, p.T1030I and p.W1140G, rendered cells nearly as sensitive as those expressing p.L35P, with survival percentages of 58% and 64%, respectively, while the expression of several other variants (p.P8L, p.K18R, p.R37H, p.H46Y, p.L947F, p.L947S and p.L1119P) only resulted in a moderate, but still significant sensitivity to PARPi (~76-86% cell survival). For a more direct assessment of HR competency, the CRISPR-*LMNA* HR assay (45) was used to further characterize the effects of 18 selected *PALB2* variants on HR. Consistently, p.T1030I and p.W1140G exhibited substantially reduced HR (>65% reduction in HR when compared to WT *PALB2*), followed by p.Y28C and p.R37H (60-65% reduction in HR when compared to WT *PALB2*), whereas other variants (p.P8L, p.L947F, p.L947S and p.G1043A), showed more intermediate phenotypes (40-60% reduction in HR when compared to WT *PALB2*).

In our recent study (39), a large number of *PALB2* truncating variants was included (p.Q60Rfs, p.S172Gfs, p.E230X, p.Y409X, p.L531Cfs, p.Y551X, p.E669Gfs, p.C882Wfs, p.Q988X, p.P1009Lfs, p.W1038X and p.Y1183X), as well as several variants that were classified as benign by ClinVar (p.Q559R, p.E672Q, p.V858=, p.P864S, and p.G998E) (Table 2). Our functional analysis relied on the stable integration of *PALB2* cDNA at a safe-harbor locus in *Palb2* knockout (KO) mouse embryonic stem (mES) cells and its subsequent expression from a relatively weak promotor (39). Such a strategy avoids differences in *PALB2* expression following siRNA-mediated knockdown and reduces possible artefacts that may arise from transient overexpression of *PALB2* cDNA. While benign variants had only a moderate or no impact on HR (<20% reduction in HR when compared to WT *PALB2*), all truncating variants strongly impacted HR (>89% reduction in HR when compare to WT *PALB2*). Moreover, 48 *PALB2* VUS were analyzed, of which the expression of 15 VUS (i.e. p.L24S, p.Y28C, p.L35P, p.R37H, p.W912G, p.G937R, p.I944N, p.L947S, p.L961P, p.L972Q, p.T1030I, p.I1037T, p.G1043D, p.L1070P and p.L1172P), strongly abrogated PALB2 protein function, with HR being decreased by 55-93% in DR-GFP assays. The same variants also resulted in cellular sensitivity to PARPi (Table 2) (39).

Table 2. Complete list of human *PALB2* variants analyzed in five independent studies.

Study	Park et al. 2014	Foo et al. 2017	Boonen et al. 2019						Wiltshire et al. 2019			Rodrigue et al. 2019		
Nr. of variants	4	5	70						91			47		
Nr. of benign controls (ClinVar) + functional range from main assay	0	0	5 (80-95% in DR-GFP)						6 (88-128% in DR-GFP)			3 (102-104% in PARPi)		
Nr. of damaging controls + functional range from main assay	0	0	12 (6-11% in DR-GFP)						7 (12-48% in DR-GFP)			1 (48% for p.L35P in PARPi)		
Cell type	U2OS	B400	mES				HeLa	U2OS	B400	HeLa	U2OS	HeLa		U2OS
Protein change	**DR-GFP (%)**	**DR-GFP (%)**	**DR-GFP (%)**	**PARPi (%)**	**Cispl. (%)**	**G2>M (%)**	**RAD51 (%)**	**LMNA (%)**	**DR-GFP (%)**	**RAD51 (%)**	**LMNA (%)**	**PARPi (%)**	**RAD51 (%)**	**LMNA (%)**
p.P4L												87	NT	NT
p.P4S			98	73	NT	NT	NT	NT						
p.P5S			62	96	NT	NT	NT	NT						
p.P8L									142	NT	NT	76	68	~ 60
p.L9H									116	NT	NT			
p.K18R		~ 75	100	94	84	87	NT	NT				82	103	~ 87
p.L21A												NT	NT	NT
p.L24S*			21	55	NT	NT	NT	NT	34	~ 11	< 5			
p.Y28C*		~ 35	33	22	NT	NT	NT	NT	96	NT	NT	92	64	~ 37
p.K30N		~ 105							92	NT	NT	97	NT	NT
p.T31I			97	102	NT	NT	NT	NT						
p.L32V												87	NT	NT
p.R34C									98	NT	NT			
p.R34H									100	NT	NT			
p.L35P*		~ 5	10	10	26	261	NT	NT	16	~ 4	< 9	48	2	~ 5
p.R37C									94	NT	NT			
p.R37H*		~ 78	45	68	83	176	NT	NT	82	NT	NT	86	71	~ 38
p.R37S									90	NT	NT			
p.E42K			105	95	90	102	NT	NT						
p.H46Y												76	108	~ 82
p.Q60Rfs			9	11	NT	NT	NT	NT						
p.P65L												86	NT	NT
p.I76V												93	NT	NT
p.V78I									132	NT	NT	92	NT	NT
p.K81R												93	NT	NT
p.E94K												94	NT	NT
p.G115V												107	NT	NT
p.T124I												109	NT	NT
p.S133T									88	NT	NT			
p.D134N			91	93	98	55	NT	NT						
p.L169I									94	NT	NT	103	80	~ 77
p.S172Gfs			9	14	NT	NT	NT	NT						
p.P207R												101	NT	NT
p.P210L			85	103	NT	NT	NT	NT	130	NT	NT	108	NT	NT
p.E230X			7	18	NT	NT	NT	NT						
p.Q251X									16	NT	NT			
p.T300I									72	NT	NT			
p.I309V									116	NT	NT			
p.T317P												109	NT	NT
p.S319Y									94	NT	NT	110	79	~ 83
p.L337S			87	116	NT	NT	NT	NT	106	NT	NT			
p.S382I									146	NT	NT			
p.T397I									98	NT	NT			
p.T397S									104	NT	NT			
p.Y408H			92	109	93	80	NT	NT	98	NT	NT			
p.Y409C									80	NT	NT			

Table 2. Continued

Study	Park et al. 2014	Foo et al. 2017	Boonen et al. 2019						Wiltshire et al. 2019			Rodrigue et al. 2019		
Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cispl. (%)	G2>M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
p.Y409X			8	17	NT	NT	NT	NT						
p.T413K									100	NT	NT			
p.S417Y			72	84	NT	NT	NT	NT	144	NT	NT			
p.S474N									102	NT	NT			
p.D498Y			94	74	NT	NT	NT	NT						
p.K515R			75	121	NT	NT	NT	NT						
p.L531Cfs			8	24	NT	NT	NT	NT						
p.S534A									94	NT	NT			
p.Y551X	NT		8	11	20	245	NT	NT	20	NT	NT			
p.Q559R			95	118	NT	NT	NT	NT	100	NT	NT			
p.S578G									104	NT	NT			
p.D616G									88	NT	NT			
p.D616H									108	NT	NT			
p.L622P			77	65	NT	NT	NT	NT						
p.E669Gfs			7	19	NT	NT	NT	NT						
p.E672Q			80	104	NT	NT	NT	NT	88	NT	NT			
p.T706I			87	78	NT	NT	NT	NT						
p.P707L			83	88	101	94	NT	NT						
p.D715Efs									12	NT	NT			
p.N716K									106	NT	NT			
p.T734S									138	NT	NT			
p.K745E									98	NT	NT			
p.L763F									92	NT	NT			
p.V858=			84	74	NT	NT	NT	NT						
p.P864S			86	87	NT	NT	NT	NT	128	NT	NT	102	90	~ 90
p.S865P			100	78	NT	NT	NT	NT						
p.G866V									112	NT	NT			
p.D871G			84	115	87	117	NT	NT	90	NT	NT			
p.C882Wfs			6	29	NT	NT	NT	NT						
p.W912G*			7	8	NT	NT	5	8						
p.V919I									96	NT	NT			
p.D927A			76	86	NT	NT	NT	NT						
p.L931P									100	NT	NT			
p.L931R			106	95	112	80	NT	NT	72	NT	NT			
p.V932M									98	NT	NT	102	84	~ 95
p.C933Y									76	NT	NT			
p.L936S									100	NT	NT			
p.G937E									88	NT	NT			
p.G937R*			17	26	NT	NT	63	25						
p.L939W	~ 85		60	91	102	218	NT	NT	96	NT	NT	97	NT	NT
p.E940G			63	81	NT	NT	NT	NT						
p.I944N*			7	15	NT	NT	NT	NT	30	~ 2	< 9			
p.L947F									74	NT	NT	77	75	~ 55
p.L947S*			30	24	NT	NT	78	47	80	NT	NT	82	69	~ 41
p.S951F									92	NT	NT			
p.S955R												103	NT	NT
p.Q958P									124	NT	NT			
p.L961P*			7	8	27	280	13	2						
p.I966T			74	79	NT	NT	NT	NT						
p.I966V												101	NT	NT
p.L972Q*			14	13	NT	NT	NT	NT						
p.Q988X			7	15	NT	NT	NT	NT						
p.G998E			95	97	80	129	NT	NT	120	NT	NT	104	94	~ 82
p.P1009Lfs			6	25	NT	NT	NT	NT						
p.A1017T												98	NT	NT

Table 2. Continued

Study	Park et al. 2014	Foo et al. 2017	Boonen et al. 2019						Wiltshire et al. 2019			Rodrigue et al. 2019		
Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cispl. (%)	G2>M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
p.E1018D			86	84	112	73	NT	NT				95	NT	NT
p.V1019A									128	NT	NT			
p.G1021R									80	NT	NT			
p.A1025R			18	24	NT	369	NT	NT				NT	NT	NT
p.A1025T									78	NT	NT	109	NT	NT
p.T1030I*	NT		15	15	NT	NT	NT	NT	60	NT	NT	58	44	~ 24
p.I1037T*			39	52	NT	NT	NT	NT						
p.W1038X			7	12	NT	NT	NT	NT						
p.L1040S			77	100	NT	NT	NT	NT						
p.K1041T												98	NT	NT
p.G1043A									98	NT	NT	87	86	~ 47
p.G1043D*			11	10	37	276	56	22						
p.Q1044H									94	NT	NT			
p.I1051S			91	109										
p.S1058P			96	85	119	109	NT	NT						
p.C1060S									104	NT	NT			
p.Y1064C			101	87	129	100	NT	NT	88	NT	NT			
p.L1070P*			23	57	NT	NT	NT	NT	34	~ 4	< 5			
p.S1075G												99	NT	NT
p.E1083D									102	NT	NT			
p.S1084L												94	NT	NT
p.P1088S									94	NT	NT			
p.I1093T									80	NT	NT	98	96	~ 92
p.T1099R									102	NT	NT			
p.S1102R									154	NT	NT	97	NT	NT
p.V1103L									100	NT	NT			
p.V1103M									154	NT	NT			
p.V1105A												89	NT	NT
p.L1107P									92	NT	NT			
p.Y1108Sfs									12	NT	NT			
p.P1111A			103	88	NT	NT	NT	NT						
p.Q1114H									98	NT	NT	92	NT	NT
p.L1119P			94	104	NT	NT	NT	NT	98	NT	NT	84	89	~ 68
p.G1121Vfs									12	NT	NT			
p.V1123M			76	103	NT	NT	NT	NT						
p.G1135E									98	NT	NT			
p.W1140G*									98	NT	NT	64	66	~ 35
p.L1143H			70	98	NT	NT	NT	NT						
p.L1143P	~ 80								132	NT	NT	109	NT	NT
p.G1145R									104	NT	NT			
p.G1147R									96	NT	NT			
p.L1150R									150	NT	NT			
p.W1159L			91	108	NT	NT	NT	NT						
p.S1160P			92	103	NT	NT	NT	NT						
p.W1164C			81	96	NT	NT	NT	NT	100	NT	NT			
p.S1165L									98	NT	NT			
p.G1166Vfs									12	NT	NT			
p.T1167I									98	NT	NT			
p.H1170Y									94	NT	NT			
p.L1172P*			13	17	NT	NT	NT	NT						
p.G1174R			91	103	NT	NT	NT	NT						
p.I1180T			82	99	NT	NT	NT	NT	72	NT	NT	106	NT	NT
p.Y1183C			71	109	NT	NT	NT	NT						
p.Y1183X			11	10	20	345	NT	NT	48	NT	NT			

All variants are indicated at the protein level (i.e., protein change). Nucleotide annotations for each variant are available in the published manuscript, where nucleotide numbering reflects Human Genome Variation Society (HGVS) nomenclature and cDNA number +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (*PALB2* NM_024675.3). The initiation codon is codon 1. Results from DR-GFP, PARPi sensitivity, cisplatin sensitivity, RAD51 foci and G2/M checkpoint assays are shown. Only data taken from bargraphs and experiments in the context of full-length PALB2 protein was used for this table. Truncating, benign (ClinVar), synthetic missense variants and strongly damaging VUS (with >50% reduced activity), are indicated in red, green, orange and with a red * in the 'protein change' column, respectively. NT stands for 'not tested'.

Effect of VUS in PALB2's CC domain on the BRCA1-interaction and HR

Formation of the PALB2-BRCA1/2-RAD51 complex is crucial for delivering RAD51 monomers to RPA-coated ssDNA overhangs and promoting strand invasion during HR (10-12,16). Variants that affect PALB2's interaction capability with BRCA1 or BRCA2 are therefore predicted to impact HR. Here we first discuss the implication of variants in PALB2's CC domain (amino acids 9 to 44) (Fig. 1). Initially it was shown by two independent studies that exchange of PALB2's CC domain residues p.L21, p.Y28 or p.L35 by an alanine (11), or p.L21 or p.L24 by a proline (12), indeed impaired HR by abolishing the interaction between PALB2 and BRCA1. Consistently, the patient-derived p.L35P missense variant in *PALB2* was more recently shown to impair the interaction with BRCA1, thereby strongly reducing HR (40). This variant was taken along by the three recent studies which all confirmed these findings (39,42,43). A similar defect in HR was observed for p.L24S, which was also attributable to an impairment in the interaction with BRCA1 (39,43). Interestingly, cycloheximide chase experiments to monitor protein stability suggested that variants that fail to interact with BRCA1 (p.L24S and p.L35P) enhanced the stability and consequently the levels of PALB2 protein (43). Consistent with this result, we and others also detected slightly higher protein levels for variants that failed to interact with BRCA1 (i.e. p.L24S, p.Y28C and p.L35P) (39,40,43). As the CC domain regulates PALB2 self-interaction in addition to the interaction with BRCA1, it is possible that an inability of PALB2 to interact with BRCA1 creates a shift towards the formation of PALB2 oligomers (53,54). Such complexes may shield PALB2 from ubiquitination-dependent degradation (55), leading to higher protein levels.

The consistency between the different studies (39,42,43), was challenged by the analysis of p.R37H, which has previously been shown to represent a variant whose expression only moderately impacts protein function (40). p.R37H was shown to reduce HR only by ~20% (40,43). Accordingly, the analysis by Foo et al. showed that p.R37H did not affect the interaction with BRCA1 (40). In contrast, Rodrigue et al. identified p.R37H as a variant whose expression led to a significant reduction in PALB2 function, both in PARPi sensitivity assays as well as the CRISPR-LMNA HR assay, with 60% reduced activity in the latter assay (42).

However, the mechanism for the reduced functionality was unclear as mammalian two-hybrid assays and laser micro-irradiation experiments suggested that this variant interacted normally with BRCA1 and was recruited to DNA damage sites, respectively. Although we reported a similar impact on HR in DR-GFP assays for this variant (55% reduction in HR when compared to WT *PALB2*) (39), we observed a partial loss of the PALB2-BRCA1 interaction in immunoprecipitation experiments, as well as the recruitment of PALB2 to sites of DNA damage induced by laser micro-irradiation (39). Thus, while all four studies consistently show the impact of p.R37H on HR, the discrepancy in the mechanistic explanation warrants further investigation of this particular variant.

Effect of VUS in PALB2's WD40 domain on protein stability and HR

In addition to the CC domain, which mediates the interaction with BRCA1, the WD40 domain of PALB2 (amino acids 853 to 1186) (Fig. 1), mediates interactions with other core HR proteins such as BRCA2 and RAD51. In our study, many damaging variants were identified in this functional domain (p.W912G, p.G337R, p.I944N, p.L947S, p.L961P, p.L972Q, p.T1030I, p.I1037T, p.G1043D, p.L1070P and p.L1172P) (39). Since all these variants exhibited strongly reduced protein expression levels, the effect on the interaction of PALB2 with other HR factors was not examined. Importantly, reverse transcription-quantitative (RT-q)PCR analysis indicated that these variants did not affect expression at the mRNA level (39), suggesting that the low abundance of PALB2 protein is likely the result of protein instability. In contrast, Rodrigue et al. performed PALB2-BRCA2 immunoprecipitation assays for damaging variants in the WD40 domain (p.L947F, p.L947S, p.T1030I, p.G1043A, p.L1119P and p.W1140G), although they similarly detected lower expression levels for these variants (42). Not surprisingly, all six variants appeared to impair the interaction with BRCA2. As these variants are scattered throughout the WD40 domain, it seems likely that they represent unstable variants rather than variants that impair specific binding sites for BRCA2. Likewise, Wiltshire and colleagues showed that the p.I944N and p.L1070P variants both decreased the interaction with BRCA2, as well as with BRCA1 (43). As the interaction motif for BRCA1 lies in PALB2's N-terminal CC domain, and not the WD40 domain in which these variants are present, these reduced interactions are more likely the result of reduced PALB2 protein stability. Although we identified several damaging variants in the WD40 domain, only the synthetic missense variant p.A1025R displayed normal expression levels, while having a major impact on HR (82% reduction in HR when compared to WT *PALB2*) (39). These results are in line with the fact that this variant impairs the PALB2-BRCA2 interaction, as shown previously by several studies (42,43,52).

In addition to the observed protein instability, it has been suggested that mis-localization of the PALB2 protein in the cytoplasm may provide an explanation for the reduced

PALB2 functionality observed for a number of variants in the WD40 domain (39,41,43). For instance, for p.I944N, Wiltshire and colleagues showed that this variant prevented nuclear localization of PALB2 and that it is retained in the cytoplasm. They observed a similar mis-localization for p.L1070P, albeit to a lesser extent. Rodrigue and colleagues additionally identified p.L947F, p.L947S, p.T1030I, p.G1043A, p.L1119P and p.W1140G as variants causing mis-localisation of PALB2. All these variants impaired PALB2 recruitment to laser-induced DSBs, an effect that was also observed for p.Y28C and p.L35P (42). However, p.Y28C and p.L35P, which both reside in the CC domain, did not negatively impact PALB2's nuclear localisation. Thus, variants in the WD40 domain that result in PALB2 instability may be signalled for degradation in the cytoplasm, providing an explanation for how such variants could impact PALB2-dependent HR.

Limitations of current assays used for the functional analysis of VUS in *PALB2*

A reasonable number of overlapping VUS was analyzed by three recent studies (39,42,43). This allows for a head-to-head comparison of the outcome of the different functional analysis, as well as the important aspects of the different experimental approaches, such as the model cell line, complementation by transient overexpression or stable expression, and the use of KO or knockdown cell lines. These differences may explain certain discrepancies, which we discuss below on the basis of several variants that were functionally characterized.

Overexpression of the *PALB2* cDNA may underestimate the functional effect of some variants. For instance, the FA-associated p.Y1183X *PALB2* variant, is located three amino acids from the end of the protein and can lead to the expression of a near full-length PALB2 protein. Stable expression of this variant impaired the HR efficiency in mES cells to a similar extent as all other truncating variants positioned throughout the gene (i.e., HR being reduced by 89-94%) (39). However, it is feasible that cDNA-based overexpression of this variant can partially rescue HR. This may have occurred in the study by Wiltshire and colleagues in which p.Y1183X reduced the HR efficiency in *Palb2*-deficient B400 mouse mammary cells by 52%, in comparison to a ~84% reduction observed for other truncating *PALB2* variants scattered throughout the gene (43). Accordingly, Rodrigue and colleagues noted that there are indeed differences in expression between variants after transient overexpression. Moreover, they showed that exogenous *PALB2* is greatly overexpressed in comparison to endogenous *PALB2* (42). Thus, we may need to take caution when variants are functionally characterized by transient overexpression, as damaging variants may still exhibit residual activity under these conditions. In fact, when we compare other overlapping variants among the three recent studies tested in DR-GFP assays (n = 26) (39,43) and PARPi sensitivity assays (n = 14) (39,42), functional defects are almost always smaller when assessed by transient overexpression compared to stable integration and expression (Fig. 3a-b). This is particularly

striking in the case of variants such as p.Y28C, p.R37H, p.L947S and p.T1030I, which may still exhibit residual activity. Consequently, this effect may lead to an underestimation of the HR defects that these variants can cause and may explain the fairly low correlation (R^2 = *~0.58*) between results from assays with transient overexpression versus stable integration and expression (Fig. 3c-d). However, this hypothesis is contradicted by the very good correlation (R^2 = *~0.91*) (Fig. 3e-f) between the effects of overlapping variants in DR-GFP and CRISPR-LMNA HR assays (n = 9) (39,42), which relied on stable and transient expression of *PALB2*, respectively. Although this result can be explained by a slightly more effective siRNA-based knockdown of endogenous *PALB2* in the U2OS cells used for the CRISPR-LMNA HR assays, it is also possible that stable versus transient expression, in a specific cellular background, impacts the outcome of the functional assays. Further research is therefore be needed to resolve these issues.

Similar to transient overexpression, *PALB2* complementation after knockdown of the gene (versus the use of KO cells), could in theory also result in an underestimation of the effects of some variants. This is because the knockdown is often incomplete, resulting in residual expression of wildtype *PALB2* in the presence of exogenously expressed *PALB2* carrying a variant. If the *PALB2* variant affects PALB2 protein function, this effect may be obscured by the presence of wildtype PALB2 protein. Also, the knockdown efficiency can differ between experiments, resulting in variability in the measured functional effects. On the other hand, with regard to the KO of genes in general, it is possible that cells can undergo adaptions in order to survive. It is possible that such adaptions can influence the functional readout that is used.

As all three recent studies employed a cDNA-based complementation approach (39,42,43), another disadvantage, specifically when analyzing truncating variants, is the absence of nonsense-mediated mRNA decay. Hypothetically, the expression of a partially functional truncated protein might mask the severe impact on protein function of such variants observed in the presence of nonsense-mediated mRNA decay, which would otherwise abrogate protein expression. A complementation method based on the use of a bacterial artificial chromosome (BAC) that contains the complete gene-of-interest would allow for inclusion of effects originating from nonsense-mediated mRNA decay. This is important, as such processes by themselves may enhance the risk for cancer and constitute an alternative mechanism for reduced protein function.

With regard to the differences in outcome between the three recent studies on *PALB2* VUS (39,42,43), one may also question whether these may originate from the use of human and mouse model cell lines. For instance, we showed that complementation of *Palb2*^{KO} mES cells with human *PALB2* cDNA resulted in a partial rescue of the HR defect (i.e., ~68% HR compared to *Trp53*^{KO} still expressing mouse *Palb2*) (39). Although it cannot be excluded that

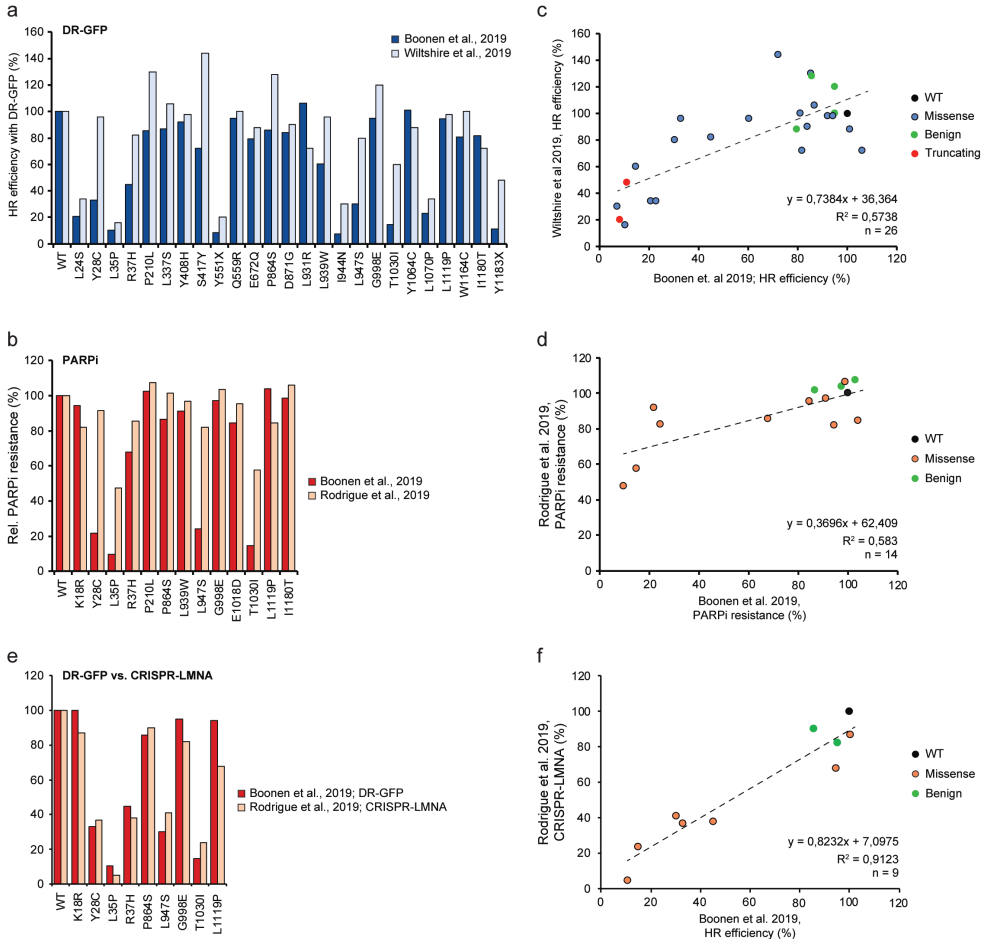

Figure 3. Comparison and correlation between DR-GFP- and PARPi-based HR assays from three different studies. a Bar graph comparing results from DR-GFP-based functional assays for 26 overlapping *PALB2* variants from studies by us and Wiltshire et al. (39,43). Mean percentages of GFP-positive cells relative to wild type *PALB2* (WT) are shown, with cells expressing WT *PALB2* being set to 100%. **b** Bar graph comparing results from PARPi-based functional assays for 14 overlapping *PALB2* variants from studies by us and Rodrigue et al. (39,42). Mean percentages of viability relative to WT *PALB2* are shown, with cells expressing WT *PALB2* being set to 100%. **c** Scatter plot showing the correlation between the results from our study and Wiltshire et al. as shown in 'a' (39,43). The color of the datapoints corresponds to the different variants/conditions: wild type (black), benign based on ClinVar (green), truncating (red), VUS (blue). **d** Scatter plot showing the correlation between the results from our study and Rodrigue et al. as shown in 'b' (39,42). The color of the datapoints corresponds to the different variants/conditions: wild type (black), benign based on ClinVar (green), VUS (orange). **e** Bar graph comparing results from DR-GFP- and CRISPR-LMNA-based HR assays for 9 overlapping *PALB2* variants from studies by us and Rodrigue et al. (39,42). Mean percentages of GFP- or mRuby-positive cells relative to WT *PALB2* are shown as in 'a'. **f** Scatter plot showing the correlation between the results from our study and Rodrigue et al. as shown in 'e' (39,42). The color of the datapoints is as shown in 'd'.

this is due to different expression levels of ectopic human *PALB2* compared to endogenous mouse *Palb2*, it is also possible that this is due to the limited homology between mouse and human *PALB2* (~59% identical and 70% similar in protein sequence). Consequently, the functional effect of some variants may be missed and this could affect the reliability of testing human variants in a mouse cell background. Nonetheless, it should be noted that so far damaging missense variants in *PALB2* have only been observed in the well conserved CC and WD40 domains, which both exhibit ~82.5% identical and ~91.5% similar protein sequence. This makes it unlikely that *PALB2* variants that have been identified as damaging in these domains in mouse cell-based assays, are not so in a human cell-based setup. Indeed, we have observed similar effects on HR for a number of VUS (p.W912G, p.G937R, p.L947S, p.L961P and G1043D) in human and mouse cell-based assays (39) .

Functional characterization of VUS in *PALB2* using checkpoint control as a read-out

Besides a critical role in promoting HR, several studies have implicated *BRCA1*, *BRCA2* and *PALB2* in DNA-damage-induced checkpoint control (56-58). Consistently, it was shown that G2/M checkpoint maintenance after IR is compromised in $Trp53^{KO}/Palb2^{KO}$ mES cells, an effect that could be rescued by expressing WT human *PALB2* (Fig. 2) (39). Interestingly, *PALB2* variants that show LOF in HR, were unable to maintain an efficient G2/M checkpoint response (p.L35P, p.L961P, p.A1025R and p.G1043D). The fact that p.L35P and p.A1025R, which are unable to interact with BRCA1 and BRCA2, respectively, were among these variants, suggests that both interactions are key to PALB2's role in regulating G2/M checkpoint control. Although checkpoint regulation could be a distinct function of *PALB2*, another possibility is that the observed defects in G2/M checkpoint maintenance could stem from defective HR. Given that a defect in HR likely leads to elevated levels of unrepaired DNA breaks, it may seem counterintuitive that G2/M checkpoint maintenance is reduced under these conditions, unless compensatory pathways take over to complete DNA repair and allow for continued progression through the cell cycle. In line with such a scenario, an inverse correlation has been observed between HR activity and a-NHEJ mediated by POLQ (59). This indicates that a-NHEJ may act as a compensatory pathway for PALB2-dependent HR. Indeed, in HR-deficient ovarian cancer cell lines POLQ was selectively upregulated, whereas restoration of HR brought back POLQ expression to normal levels (59). Based on these findings we speculate that when HR is compromised due to PALB2 LOF, activation of a-NHEJ potentially affects G2/M checkpoint maintenance in response to DNA breaks.

Control of ROS and replication stress as potential readouts for the functional analysis of VUS in *PALB2*

PALB2 has also been reported to play a role in controlling the reactive oxygen species (ROS) levels in human cells (60), which may constitute another tumor suppressive function. PALB2 suppresses ROS levels in a manner dependent on its interaction with the ubiquitin ligase KEAP1. KEAP1 functions as a cysteine-rich oxidative stress sensor, which under normal conditions, binds to and targets the antioxidant transcription factor NRF2 for degradation (60). As PALB2 bears a highly conserved ETGE-type KEAP1-binding motif (amino acids 88 to 94), that is identical to that of NRF2, PALB2 can competitively impede the inhibitory KEAP1-NRF2 interaction. Therefore, PALB2 is believed to promote NRF2 accumulation, enhance antioxidant gene expression and reduce the burden of oxidative stress. However, the truncating p.Y551X *PALB2* variant, which has been described to be associated with FA and breast cancer (61), still interacts with NRF2 as corroborated by Ma and colleagues (60), and consequently should be functional in the regulation of ROS levels. Furthermore, this truncated variant has been shown to be expressed in lymphoblasts of an individual with FA and is apparently not subjected to nonsense RNA-mediated decay (61). We therefore infer that the effect of impaired regulation of ROS levels by PALB2 may have no, or only a minor contribution to the development of FA and breast cancer, questioning the value of a more extensive analysis of the effect of VUS in *PALB2* on this process.

Besides playing a key role in HR, *BRCA1, BRCA2* and *RAD51* have also been implicated in replication fork protection and/or the recovery of stalled replication forks, which are processes that are critical for genome stability maintenance and cancer prevention, as well as cancer therapy responses (62-65). Intriguingly, it was recently shown that the interaction between BRCA1 and BARD1 promotes the protection of replication forks and that genetic variants in *BRCA1* that impair this interaction associate with cancer, even though they retain their function in HR (66). A mutational analysis of *BRCA2* revealed that a conserved C-terminal site involved in stabilizing RAD51 filaments, but not in loading RAD51 onto DNA, is essential for replication fork protection, but dispensable for HR. Consistently, the p.S3291A variant in this C-terminal region was shown to impair the protection of stalled replication forks, while leaving HR intact (65). RAD51, on the other hand, acts during DNA replication to facilitate fork reversal and protects nascent DNA strands from nuclease digestion, thereby promoting the recovery of stalled replication forks (67-69). It is plausible that PALB2 exhibits functions at the replication fork that are comparable to those of BRCA1, BRCA2 and/or RAD51. Indeed, it was previously shown that PALB2 mediates replication fork recovery after replication stress in human U2OS cells (70). Corroborating these findings, replication abnormalities, including a decreased/delayed origin firing and replication fork restart, have also been observed in blood lymphocytes heterozygous for the truncating p.L531Cfs *PALB2* variant (71). How PALB2

mechanistically facilitates these processes is still largely unclear and requires additional research. However, it is feasible that VUS in *PALB2* could have the potential to specifically impair such functions, as has been reported for *BRCA1* and *BRCA2* (65,66). Potentially, loss of PALB2's function in replication fork protection and/or recovery may associate with cancer. If so, replication fork maintenance may become another important readout for functional analysis of *PALB2* VUS.

In silico approaches predicting the functional impact of VUS are mostly unreliable

Especially with the vast accumulation of identified VUS (72,73), a variety of in silico tools, which are both publicly and commercially available, can aid in the interpretation of VUS in clinical diagnostic settings (74). However, the currently available in silico tools, such as PolyPhen-2, SIFT, MutationTaster-2, MutationAssessor, CADD and REVEL, often give rise to conflicting results and over- or underestimate the functional impact of a given variant (75,76). A systematic performance comparison between in silico prediction tools and functional assays, showed that functional assays substantially outperform every computational method examined, mostly with respect to heightened specificity (77). In this study, a panel of 26 different yeast-based complementation assays were used to measure the impact of 179 variants on 22 human disease genes. Remarkably, of the 64 non-disease-associated variants tested, 36% was predicted to be deleterious by PolyPhen, as opposed to only 13% being classified as deleterious by these functional assays (77). This high rate of false predictions is in agreement with recent data from us and Rodrigue et al., showing that in silico prediction tools all strongly overpredicted the percentage of deleterious variants in *PALB2* (39,42), Consistently, these studies observed a poor correlation between results from DR-GFP assays and predictions by CADD (R^2 = *0.08*) or REVEL (R^2 = *0.11*) (39), and between results from PARPi sensitivity assays and M-CAP (R^2 = *0.33*), VEST (R^2 = *0.07*) or REVEL (R^2 = *0.27*) (42). Due to this lack of consistency and poor performance, computational predictions are not considered strong evidence for or against pathogenicity (74). Instead, functional assays seem to represent the best strategy for overcoming the VUS challenge, as they currently constitute the strongest evidence for the functional impact of rare variants. Moreover, for genes such as *BRCA1* and *BRCA2*, for which functional assays are more established, a functional read-out such as HR can be used to improve existing computational prediction tools. In a recent study by Hart et al., the measured HR efficiency for 248 *BRCA1* and 207 *BRCA2* variants was used to recalibrate 40 in silico algorithms (78). Optimized thresholds based on such functional data significantly improved the accuracy of many of these algorithms. However, optimised algorithms for one gene may perform poorly when applied to another gene. This is perhaps not surprising as each functional domain may harbour different sensitivities to the effects of

damaging variants, explaining why different gene-specific features are important for the accuracy of in silico predictions.

Perspective on high-throughput functional analysis of *PALB2* variants

The identification of VUS has increased drastically due to the global build-up in genetic testing (79), leading to major challenges in the clinical management of carriers. To emphasize the vast number of genetic variants that are identified, 4.6 million missense variants have recently been reported in ~140000 exomes and genomes in the Genome Aggregation Database (gnomAD) (72,73) and 99% of these variants are rare with a minor allele frequency of <0.005 (80). Variant interpretation at such a scale, can currently only be addressed with computational prediction tools. However, as mentioned above, the existing tools often provide conflicting results, where functional impact is mostly overpredicted (39,77). Thus, the accelerated rate of VUS discovery makes a one-at-a-time, or even semi high-throughput, approach for functional analysis infeasible. Furthermore, as these strategies are often time-consuming, the individual in which a variant was found may not be able to take advantage of it in time.

An ambitious goal for the future is that the effect of every possible nucleotide substitution, perhaps initially only in clinically actionable genes (81,82), is functionally measured using high-throughput assays. For instance, for *PALB2* specifically, as of June 2020, 1612 distinct VUS have been reported in ClinVar. This number already makes a one-at-a-time functional analysis approach extremely challenging. High-throughput assays (i.e. multiplexed assays), aimed to address every nucleotide change in an entire gene in single experiments may provide a solution. Indeed, a saturation CRISPR/Cas9-based editing approach in haploid human HAP1 cells allowed for the assessment of more than 95% of all possible single-nucleotide variants (SNVs) in 13 exons of *BRCA1* that encode for its RING and BRCT domains (83). Importantly, this setup allowed for the functional analysis of variants in their endogenous genomic context and using cell survival as a read-out, the effect of nearly 4000 single-nucleotide variants corroborated established assessments on protein function. Furthermore, a multiplex homology-directed repair assay, which relied on stable integration of a *BRCA1* cDNA variant library, enabled the functional characterization of 1056 missense variants in the first 192 residues of *BRCA1* (38). We expect that such assays will be extended to analyzing variants in genes such as *PALB2* in the near future, ultimately leading to the development of a variant map that shows the impact of all possible *PALB2* variants on HR.

In addition to examining cell survival and HR for *PALB2* in a high-throughput setup, another more general readout might be to measure the steady-state protein abundance. Recent results from functional assays have shown that variants in PALB2's WD40 domain tend to destabilize PALB2 (39), a mechanism of protein inactivation that is in agreement with studies showing that ~75% of pathogenic variation is thought to disrupt thermodynamic stability and

alter protein levels (84-86). Therefore, high-throughput assessment of PALB2 variant protein abundance, by employing techniques such as VAMP-seq (84) or Stable-seq (87), may also prove to be highly suitable for detecting *PALB2* variants that affect protein function. Nonetheless, although such high-throughput assays provide much potential for interpreting the large number of VUS that are being identified, it should also be noted that developing variant libraries, optimizing experimental setups, and analyzing the large amount of sequencing data, can still be prohibitively time and resource intensive.

Towards the functional analysis of *PALB2* VUS in RNA splicing

It is important to note that all functional studies on VUS in *PALB2* discussed in this review (39-43), were based on expression of *PALB2* cDNAs and are therefore not suitable to assess the functional impact of *PALB2* variants that affect RNA splicing. In silico splice site prediction tools can predict the effect of variants on potential splice sites relatively well (74), but they do not provide conclusive evidence for altered splicing. One option to assess the effect of variants on splicing, is to use a minigene construct that contains a genomic segment encompassing the variant along with flanking intronic sequences (88). After transient transfection of the construct into human cells, the transcripts from the minigene can easily be analyzed and compared to transcripts derived from a wild-type construct. Although these assays can be carried out in many cell types and are fairly simple and fast, disadvantages are that variants are not measured in the context of a complete gene and that these assays do not permit downstream functional analysis. This is of course important since some splice variants can result in the expression of a transcript that may be (partially) functional. For instance, several exons in *PALB2* (exons 1, 2, 4, 6, 7, 9, 10 and 11-12 combined) can be skipped due to splice site variants and still result in an in-frame transcript (89). Such transcripts may still express an isoform of *PALB2* with an entire exon deleted, yet retain partial protein function. An example is the c.2586+1G>A (r.2515_2586del; p.T839_K862del) *PALB2* variant, which leads to an in-frame skip of exon 6. This variant appears to be a hypomorphic variant that still interacts with BRCA2 and, when overexpressed, still enables RAD51 foci formation (90). Additional research will be required to establish the functionality of other exon-skip variants in *PALB2.*

As of June 2020, 70 unique *PALB2* splice variants have been reported in ClinVar (involving canonical splice sites), the majority of which is classified as pathogenic or likely pathogenic. Generally, mRNA transcript and protein expression analysis combined with functional assays, may be needed to provide insight into the effect of variants in *PALB2* that are predicted to impact RNA splicing. Possibly, one could complement *PALB2* KO cells containing DR-GFP with a BAC containing the full length human *PALB2* gene. Such a method has previously been described for *BRCA1* and *BRCA2* (91-94) and would allow for the

introduction and functional analysis of splice variants in coding and non-coding regions, further improving their classification.

Towards estimating cancer risk associated with VUS in *PALB2*

Functional assays may aid in the classification of rare *PALB2* VUS, yet a major challenge will be to translate effects on PALB2 protein function into estimates for cancer risk. Recent studies on *BRCA2* have shown that pathogenic variants that confer high risk for breast and ovarian cancer completely abrogate BRCA2-mediated HR, whereas variants that result in a reduction of 50% in HR, i.e., hypomorphic variants, may only be associated with a moderate risk for breast cancer (Odds ratio ~2.5) (36,37). With regard to *PALB2*, truncating variants have been associated with an odds ratio of 7.46 (95% CI, 5.12-11.19) (28), whereas the frequently occurring p.L939W missense variant has been associated with an odds ratio of 1.05 (95% CI, 0.83 to 1.32) (95), which is in agreement with recent data from Wiltshire et al. and Rodrigue et al., showing that this variant does not impact the HR efficiency (~4% reduction in HR when compared to WT) (42,43). In contrast, results from us and Park et al., showed that this variant did impair HR to some degree (40% and 15% reduction in HR when compared to WT, respectively) (39,41). This may suggest that such a decrease in HR, may not considerably increase the risk for breast cancer. Future functional characterization of additional *PALB2* VUS, in combination with data from large case-control association studies, should allow for more conclusive correlations of odds ratios with HR efficiencies for *PALB2*, either for specific variants that occur frequently, or for variants as a group (i.e., damaging variants). Under the assumption that variants with similar levels of HR functionality confer the same level of cancer risk, so called burden-type of association analyses can be performed in large case-control studies, in which either genetic or clinical information of multiple variants, or joint frequencies of individual variants with similar HR levels will be pooled. Nonetheless, the fact that roles other than in HR (i.e., in replication fork stability/recovery) for all three major breast cancer susceptibility genes (*BRCA1*, *BRCA2* and *PALB2*) have been described (64-66,70), complicates the interpretation of VUS in these genes and their association with cancer risk. It should be noted, however, that only a few variants in *BRCA1* and *BRCA2,* have recently been implicated in the protection of replication forks, while having no impact on HR (65,66). To our knowledge, no such variants have yet been reported for *PALB2*. Although these *BRCA1* and *BRCA2* variants appear to associate with cancer, their exact risk needs to be further established.

The use of functional assays for predicting therapy response

Although healthy cells can often repair DNA damage by making use of their full repertoire of DNA repair mechanism, cells exhibiting deficiency in HR due to the presence of *PALB2* LOF

variants,become more reliable on alternative DNA repair mechanisms to survive and proliferate. Therefore, conventional treatment strategies (especially for HR-deficient tumours), have been developed to force DNA damage-induced cell death through synthetic lethal interactions. It is now well established that cancers that exhibit pathogenic variants in *BRCA1* or *BRCA2* respond well to treatment with PARPi (96,97), a therapeutic strategy that has emerged for *BRCA1*- and *BRCA2*-mutated breast and ovarian tumours (48,98,99). Consequently, it is of great importance to identify deleterious *PALB2* VUS that lead to HR deficiency and for which corresponding tumours may similarly respond to PARPi-based therapy.

With regard to the studies that functionally analysed VUS in *PALB2* (39-43), it is clear that within each study, the HR efficiency correlated extremely well with PARPi sensitivity, exhibiting a strong positive correlation in mES cells ($R^2 = 0.804$) (39) and human cell lines ($R^2 = 0.68$) (42). Similar results were obtained for sensitivity assays with cisplatin ($R^2 = 0.8313$) (39,43), a commonly-used chemotherapeutic for many cancers, including breast and ovarian cancer. Similar to that in many *BRCA1*- and *BRCA2*-associated tumours (100,101), many *PALB2*-associated breast cancers (i.e. 67%) show loss of the *PALB2* wild type allele via acquired pathogenic somatic variants, or via loss-of-heterozygosity (LOH) (102,103). Such *PALB2*-null cancers all exhibited HR deficiency, with some tumours even showing HR deficiency while the wild type allele was retained (102,103), suggesting that also alternative mechanisms for PALB2 LOF can be in play. With results from such studies in mind, findings from functional assays that show which VUS are damaging or functional, may prove to be valuable for predicting platinum- and/or PARPi-based therapy response in cancer patients that carry *PALB2* variants that abrogate HR.

Concluding remarks

Due to the accelerating pace by which genetic variants in *PALB2* are discovered, there is a strong need to determine which variants actually associate with disease causation. The combined effort to functionally characterize 155 *PALB2* genetic variants, for which clinical significance is unknown, represents a milestone in the reclassification of these variants. Classification of VUS to a category with a defined clinical significance is of great importance to carriers of a pathogenic variant. This will allow them to make an informed decision on how to manage their cancer risk, including increased surveillance or risk reducing surgery to reduce cancer incidence and/or offering testing of relatives at risk. Counselees carrying non-pathogenic variants may be discharged from intensive follow-up and avoid unnecessary risk-reducing surgery (104).

In this review, we have provided head-to-head comparisons of the different assays that were used for the functional characterization of variants in *PALB2*. These analyses are an

important starting point for the identification of variants that impact its major tumor suppressive function, which most likely is to be attributed to its role in HR, and whose defects correlate with significantly increased cancer risk. Although these assays were able to consistently determine effects of several variants on PALB2's function during HR, some differences in PALB2 function were also observed (Fig. 3), which may be attributed to the type of cDNA-based complementation approach being used. With regard to functional assays being used as clinical diagnostic tools, it is essential to combine results from functional assays that have been obtained by employing different experimental strategies (74,105,106). Moreover, most functional assays use HR as a read-out. However, if PALB2's role in checkpoint control, the regulation of cellular ROS levels and/or the maintenance of replication fork integrity may contribute to its tumor suppressive function as well, expanding the different read-outs of functional assays to cover these aspects of PALB2 function will be a must. Generally, these assays should also include the possibility of a combined mRNA and protein expression analysis in order to provide insight into the effect of variants in coding and non-coding regions of *PALB2* that are predicted to affect RNA splicing, further improving their classification.

Until more conclusive correlations between the level of impairment of protein function and associated cancer risk have been established, results from functional assays should be implemented with care when making a clinical assertion with regard to associated cancer risk and targeted therapies. In light of the increasing number of *PALB2* variants that will undoubtedly be identified in the future, this information will ultimately be crucial for clinical geneticists in selecting the appropriate strategy for clinical management of carriers of (rare) variants in *PALB2*.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

RACMB, MPGV and HvA conducted literature research and wrote the paper.

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