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Functional Characterization of *PALB2* Variants of Uncertain Significance: Toward Cancer Risk and Therapy Response Prediction

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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 (PARPi)

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PALB2 IS ESSENTIAL FOR DNA 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 signaling 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 non-homologous end joining (c-NHEJ), alternative non-homologous end-joining (a-NHEJ), single-strand annealing (SSA), break-induced replication (BIR), and homologous recombination (HR) (Ciccio and Elledge, 2010; Chapman et al., 2012). 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 (Sishe and Davis, 2017). 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 (Hustedt and Durocher, 2016). During HR, BRCA1 inhibits 53BP1 from interacting with the chromatin near the broken DNA ends (Chapman et al., 2012; Densham et al., 2016). 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 (Marini et al., 2019). Following resection, the 3'-ssDNA tails become coated by the RPA heterotrimer (Symington, 2016). 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 (Figure 1) (Xia et al., 2006; Sy et al., 2009b; Zhang et al., 2009a,b; Prakash et al., 2015). BRCA2 possesses eight highly conserved BRC repeats and a carboxy-terminal region that have been shown to bind RAD51 (Bignell et al., 1997; Wong et al., 1997; Esashi et al., 2005). 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 (Jensen et al., 2010). 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 (Luijsterburg et al., 2017). 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 (Zong et al., 2019; Callen et al., 2020). Thus, it is apparent that RAD51 loading during HR, regardless of its dependency on BRCA1, RNF168, or both, requires the action of PALB2.

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 (Nik-Zainal et al., 2016; Polak et al., 2017), revealed that mutational inactivation of the *BRCA1*, *BRCA2*, and *PALB2* genes was the most common genetic cause of the observed HR signatures (Nguyen et al., 2020), 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 (Antoniou et al., 2003, 2014), whereas bi-allelic LOF variants cause Fanconi anemia (FA) (Howlett et al., 2002; Sawyer et al., 2015). The *PALB2* gene, which is located on chromosome 16p12.2, comprises 13 exons and encodes a protein of 1186-amino acids (Figure 1), was identified in 2006 as an important BRCA2-interacting protein (Xia et al., 2006; Tischkowitz and Xia, 2010). As it has now been established that LOF variants in *PALB2* convey a similarly high risk for breast cancer as *BRCA2* LOF variants (Antoniou et al., 2003, 2014; Couch et al., 2017), *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 (Jones et al., 2009; Slater et al., 2010; Hofstatter et al., 2011; Hu et al., 2018; Yang et al., 2020).

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 (Figure 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* (Bouwman et al., 2013; Woods et al., 2016; Shimelis et al., 2017; Starita et al., 2018; Mesman et al., 2019). More recently, VUS in *PALB2* have also been characterized in a similar manner (Figures 1, 2) (Park et al., 2014; Foo et al., 2017; Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019). 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 (Kass et al., 2013; Ducey et al., 2019). 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 (Figure 2). Furthermore, *PALB2* function was assessed by exposing cells that express a *PALB2* variant

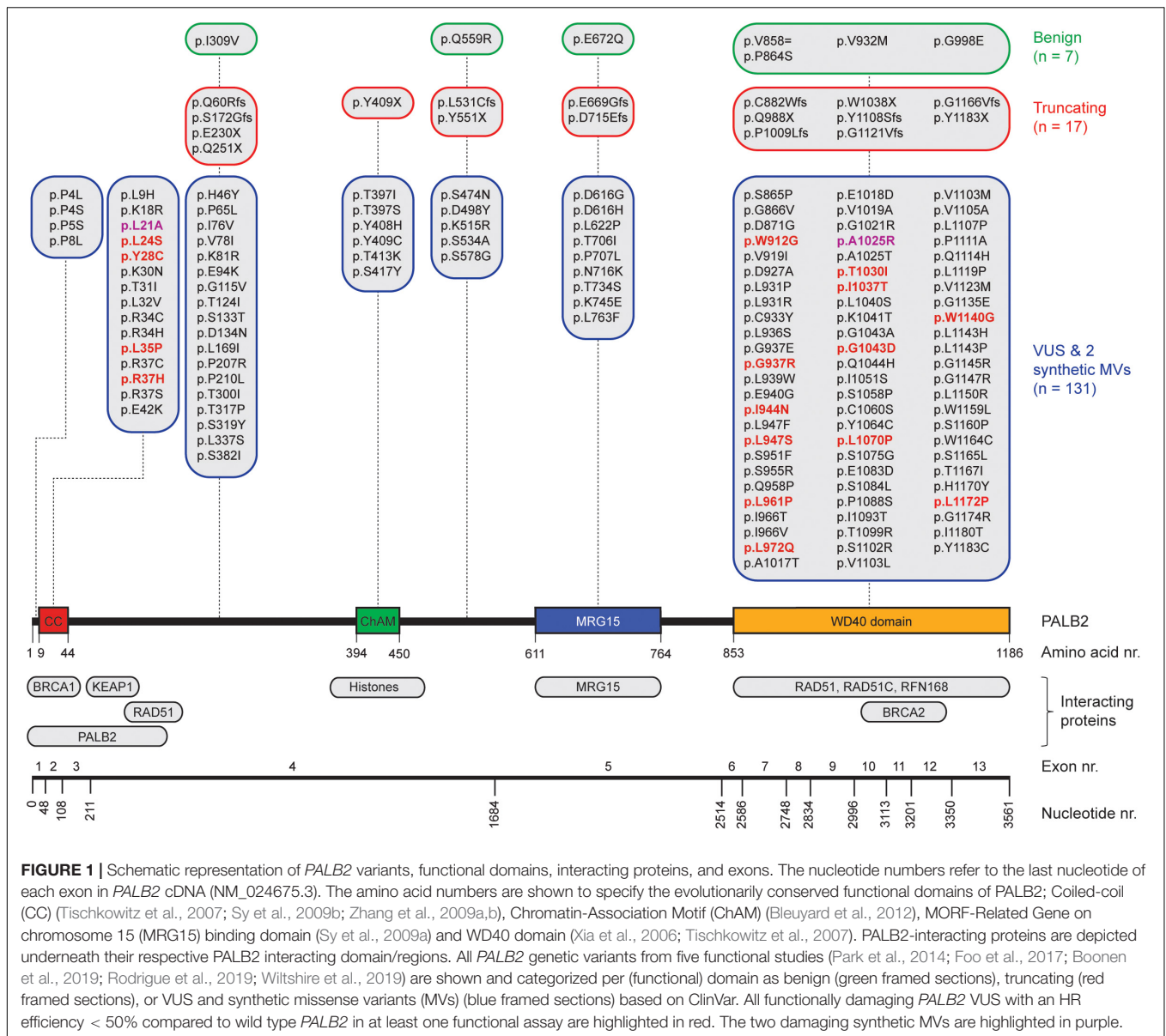
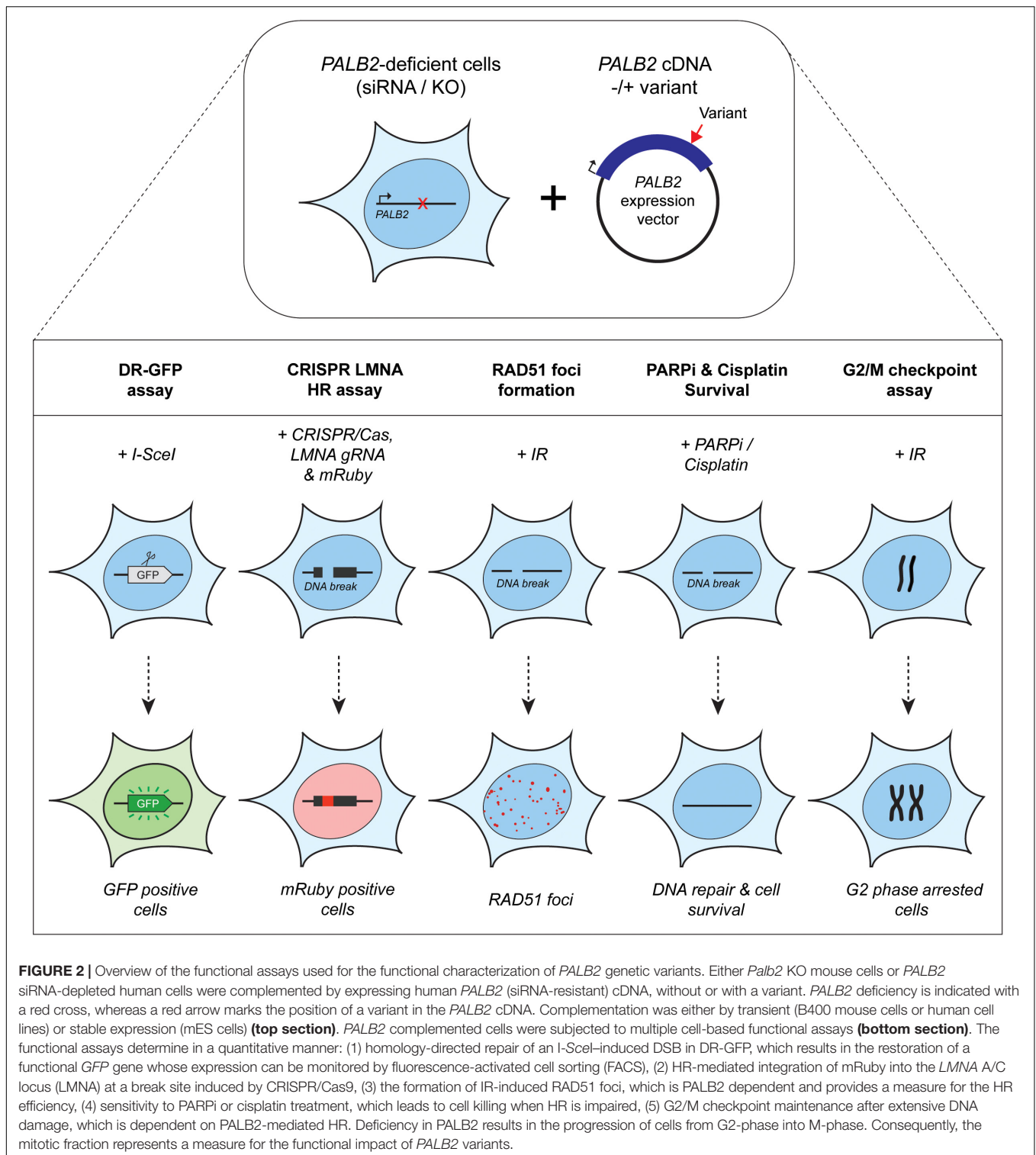


FIGURE 1 | Schematic representation of *PALB2* variants, functional domains, interacting proteins, and exons. The nucleotide numbers refer to the last nucleotide of each exon in *PALB2* cDNA (NM_024675.3). The amino acid numbers are shown to specify the evolutionarily conserved functional domains of *PALB2*; Coiled-coil (CC) (Tischkowitz et al., 2007; Sy et al., 2009b; Zhang et al., 2009a,b), Chromatin-Association Motif (ChAM) (Bleuyard et al., 2012), MORF-Related Gene on chromosome 15 (MRG15) binding domain (Sy et al., 2009a) and WD40 domain (Xia et al., 2006; Tischkowitz et al., 2007). *PALB2*-interacting proteins are depicted underneath their respective *PALB2* interacting domain/regions. All *PALB2* genetic variants from five functional studies (Park et al., 2014; Foo et al., 2017; Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019) 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.

to PARP inhibitor (PARPi) or cisplatin (Figure 2). Catalytic inhibition of PARP1 “traps” PARP1 molecules on endogenous ssDNA breaks, resulting in replication fork collapse and DSB formation (Murai et al., 2012). 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 through the FA pathway (Deans and West, 2011). Both PARPi- and ICL-induced DSBs are repaired by HR. Consequently, in the absence of HR (e.g., due to *PALB2* LOF), PARP-trapping 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 (McCabe et al., 2006; Lord and Ashworth, 2016, 2017). 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 two variants, p.L939W and p.L1143P, impaired *PALB2* function (Park et al., 2014). Lastly, since *PALB2* interacts with BRCA1 and BRCA2 to load RAD51 at sites of DSBs, co-immunoprecipitation, recruitment to laser 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 (Figure 2) (Park et al., 2014; Foo et al., 2017; Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019). A complete overview of all functional assays that were performed by three recent studies is provided in Table 1 (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019). With the above described functional assays, these studies analyzed a total of 155 different *PALB2*



variants (**Table 2**), comprising 129 VUS, 7 benign variants (as classified by ClinVar) (Landrum et al., 2014), 2 synthetic missense variants with known LOF (Oliver et al., 2009; Sy et al., 2009b) and 17 truncating variants (**Figure 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* (**Figure 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.

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
	Recruitment to laser-induced DNA damage tracks	3
	Co-immunoprecipitation	3
Rodrigue et al., 2019 (47 variants)	PARPi sensitivity (proliferation)	Complete set
	PALB2 expression blots	Complete set
	RAD51 foci number after IR	18
	RAD51 foci intensity after IR	8
	CRISPR-LMNA HR assay	18
	Recruitment to laser-induced DNA damage tracks	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
	Recruitment to laser-induced DNA damage tracks	4
	PALB2 cellular localization	4
	Mammalian two-hybrid assay (BRCA1) (1-319)	3
	Mammalian two-hybrid assay (BRCA2) (859-1186)	3
	Cyclohexamide chase	5
	Co-immunoprecipitation	6

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 analyzed by Wiltshire and colleagues (Table 2) (Wiltshire et al., 2019). 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 analyzed to validate their functional impact. The assays were mostly performed in *Palb2*-deficient B400 mouse mammary tumor 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 (Ducy et al., 2019) 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.

Rodrigue et al. (2019) first tested their set of 41 *PALB2* VUS using PARPi sensitivity assays (Table 2). Their assay was set up in HeLa cells in which endogenous *PALB2* was depleted

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
Nucleotide change	Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cisplatin (%)	G2 > M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
c.11C>T	p.P4L												87	NT	NT
c.10C>T	p.P4S			98	73	NT	NT	NT	NT						
c.13C>T	p.P5S			62	96	NT	NT	NT	NT						
c.23C>T	p.P8L									142	NT	NT	76	68	~ 60
c.26T>A	p.L9H									116	NT	NT			
c.53A>G	p.K18R		~75	100	94	84	87	NT	NT				82	103	~87
c.61T>G + 62T>C	p.L21A												NT	NT	NT
c.71T>C	p.L24S*			21	55	NT	NT	NT	NT	34	~11	<5			
c.83A>G	p.Y28C*		~35	33	22	NT	NT	NT	NT	96	NT	NT	92	64	~37
c.90G>T	p.K30N		~105							92	NT	NT	97	NT	NT
c.92C>T	p.T31I			97	102	NT	NT	NT	NT						
c.94C>G	p.L32V												87	NT	NT
c.100C>T	p.R34C									98	NT	NT			
c.101G>A	p.R34H									100	NT	NT			
c.104T>C	p.L35P*		~5	10	10	26	261	NT	NT	16	~ 4	<9	48	2	~5
c.109C>T	p.R37C									94	NT	NT			
c.110G>A	p.R37H*		~78	45	68	83	176	NT	NT	82	NT	NT	86	71	~38
c.109C>A	p.R37S									90	NT	NT			
c.124G>A	p.E42K			105	95	90	102	NT	NT						
c.136C>T	p.H46Y												76	108	~82
c.172_175delTTGT	p.Q60Rfs			9	11	NT	NT	NT	NT						
c.194C>T	p.P65L												86	NT	NT
c.226A>G	p.I76V												93	NT	NT
c.232G>A	p.V78I									132	NT	NT	92	NT	NT
c.242A>G	p.K81R												93	NT	NT
c.280G>A	p.E94K												94	NT	NT
c.344G>T	p.G115V												107	NT	NT
c.371C>T	p.T124I												109	NT	NT

(Continued)

TABLE 2 | Continued

Study		Park et al., 2014	Foo et al., 2017	Boonen et al., 2019						Wiltshire et al., 2019			Rodrigue et al., 2019		
Nucleotide change	Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cisplatin (%)	G2 > M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
c.398C>G	p.S133T									88	NT	NT			
c.400G>A	p.D134N			91	93	98	55	NT	NT						
c.505C>A	p.L169I									94	NT	NT	103	80	~77
c.514_517delTCTG	p.S172Gfs			9	14	NT	NT	NT	NT						
c.620C>G	p.P207R												101	NT	NT
c.629C>T	p.P210L			85	103	NT	NT	NT	NT	130	NT	NT	108	NT	NT
c.688G>T	p.E230X			7	18	NT	NT	NT	NT						
c.751C>T	p.Q251X									16	NT	NT			
c.899C>T	p.T300I									72	NT	NT			
c.925A>G	p.I309V									116	NT	NT			
c.949A>C	p.T317P												109	NT	NT
c.956C>A	p.S319Y									94	NT	NT	110	79	~83
c.1010T>C	p.L337S			87	116	NT	NT	NT	NT	106	NT	NT			
c.1145G>T	p.S382I									146	NT	NT			
c.1190C>T	p.T397I									98	NT	NT			
c.1189A>T	p.T397S									104	NT	NT			
c.1222T>C	p.Y408H			92	109	93	80	NT	NT	98	NT	NT			
c.1226A>G	p.Y409C									80	NT	NT			
c.1227_1231delTGTTA	p.Y409X			8	17	NT	NT	NT	NT						
c.1238C>A	p.T413K									100	NT	NT			
c.1250C>A	p.S417Y			72	84	NT	NT	NT	NT	144	NT	NT			
c.1421G>A	p.S474N									102	NT	NT			
c.1492G>T	p.D498Y			94	74	NT	NT	NT	NT						
c.1544A>G	p.K515R			75	121	NT	NT	NT	NT						
c.1592delT	p.L531Cfs			8	24	NT	NT	NT	NT						
c.1600T>G	p.S534A									94	NT	NT			
c.1653T>A	p.Y551X	NT		8	11	20	245	NT	NT	20	NT	NT			
c.1676A>G	p.Q559R			95	118	NT	NT	NT	NT	100	NT	NT			
c.1732A>G	p.S578G									104	NT	NT			
c.1847A>G	p.D616G									88	NT	NT			
c.1846G>C	p.D616H									108	NT	NT			
c.1865T>C	p.L622P			77	65	NT	NT	NT	NT						
c.2006delA	p.E669Gfs			7	19	NT	NT	NT	NT						
c.2014G>C	p.E672Q			80	104	NT	NT	NT	NT	88	NT	NT			
c.2117C>T	p.T706I			87	78	NT	NT	NT	NT						
c.2120C>T	p.P707L			83	88	101	94	NT	NT						

(Continued)

TABLE 2 | Continued

Study		Park et al., 2014	Foo et al., 2017	Boonen et al., 2019						Wiltshire et al., 2019			Rodrigue et al., 2019		
Nucleotide change	Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cisplatin (%)	G2 > M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
c.2145_2146delT	p.D715Efs									12	NT	NT			
c.2148T>A	p.N716K									106	NT	NT			
c.2200A>T	p.T734S									138	NT	NT			
c.2234A>G	p.K745E									98	NT	NT			
c.2289G>C	p.L763F									92	NT	NT			
c.2574T>C	p.V858 =			84	74	NT	NT	NT	NT						
c.2590C>T	p.P864S			86	87	NT	NT	NT	NT	128	NT	NT	102	90	~ 90
c.2593T>C	p.S865P			100	78	NT	NT	NT	NT						
c.2597G>T	p.G866V									112	NT	NT			
c.2612A>G	p.D871G			84	115	87	117	NT	NT	90	NT	NT			
c.2642_2645dupGTTG	p.C882Wfs			6	29	NT	NT	NT	NT						
c.2734T>G	p.W912G*			7	8	NT	NT	5	8						
c.2755G>A	p.V919I									96	NT	NT			
c.2780A>C	p.D927A			76	86	NT	NT	NT	NT						
c.2792T>C	p.L931P									100	NT	NT			
c.2792T>G	p.L931R			106	95	112	80	NT	NT	72	NT	NT			
c.2794G>A	p.V932M									98	NT	NT	102	84	~ 95
c.2798G>A	p.C933Y									76	NT	NT			
c.2807T>C	p.L936S									100	NT	NT			
c.2810G>A	p.G937E									88	NT	NT			
c.2809G>A	p.G937R*			17	26	NT	NT	63	25						
c.2816T>G	p.L939W	~85		60	91	102	218	NT	NT	96	NT	NT	97	NT	NT
c.2819A>G	p.E940G			63	81	NT	NT	NT	NT						
c.2831T>A	p.I944N*			7	15	NT	NT	NT	NT	30	~2	<9			
c.2841G>T	p.L947F									74	NT	NT	77	75	~55
c.2840T>C	p.L947S*			30	24	NT	NT	78	47	80	NT	NT	82	69	~41
c.2852C>T	p.S951F									92	NT	NT			
c.2865T>A	p.S955R												103	NT	NT
c.2873A>C	p.Q958P									124	NT	NT			
c.2882T>C	p.L961P*			7	8	27	280	13	2						
c.2897T>C	p.I966T			74	79	NT	NT	NT	NT						
c.2896A>G	p.I966V												101	NT	NT
c.2915T>A	p.L972Q*			14	13	NT	NT	NT	NT						
c.2962C>T	p.Q988X			7	15	NT	NT	NT	NT						
c.2993G>A	p.G998E			95	97	80	129	NT	NT	120	NT	NT	104	94	~82
c.3026delC	p.P1009Lfs			6	25	NT	NT	NT	NT						
c.3049G>A	p.A1017T												98	NT	NT
c.3054G>T	p.E1018D			86	84	112	73	NT	NT				95	NT	NT
c.3056T>C	p.V1019A									128	NT	NT			
c.3061G>A	p.G1021R									80	NT	NT			

(Continued)

TABLE 2 | Continued

Study		Park et al., 2014	Foo et al., 2017	Boonen et al., 2019						Wiltshire et al., 2019			Rodrigue et al., 2019		
Nucleotide change	Protein change	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cisplatin (%)	G2 > M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)
c.3073G>C + 3074C>G	p.A1025R			18	24	NT	369	NT	NT				NT	NT	NT
c.3073G>A	p.A1025T									78	NT	NT	109	NT	NT
c.3089C>T	p.T1030*	NT		15	15	NT	NT	NT	NT	60	NT	NT	58	44	~24
c.3110T>C	p.I1037T*			39	52	NT	NT	NT	NT						
c.3113G>A	p.W1038X			7	12	NT	NT	NT	NT						
c.3119T>C	p.L1040S			77	100	NT	NT	NT	NT						
c.3122A>C	p.K1041T												98	NT	NT
c.3128G>C	p.G1043A									98	NT	NT	87	86	~47
c.3128G>A	p.G1043D*			11	10	37	276	56	22						
c.3132A>C	p.Q1044H									94	NT	NT			
c.3152T>G	p.I1051S			91	109										
c.3172T>C	p.S1058P			96	85	119	109	NT	NT						
c.3179G>C	p.C1060S									104	NT	NT			
c.3191A>G	p.Y1064C			101	87	129	100	NT	NT	88	NT	NT			
c.3209T>C	p.L1070P*			23	57	NT	NT	NT	NT	34	~4	<5			
c.3223A>G	p.S1075G												99	NT	NT
c.3249G>C	p.E1083D									102	NT	NT			
c.3251C>T	p.S1084L									94	NT	NT	94	NT	NT
c.3262C>T	p.P1088S									80	NT	NT	98	96	~92
c.3278T>C	p.I1093T									102	NT	NT			
c.3296C>G	p.T1099R									154	NT	NT	97	NT	NT
c.3306C>G	p.S1102R									100	NT	NT			
c.3307G>C	p.V1103L									154	NT	NT			
c.3307G>A	p.V1103M												89	NT	NT
c.3314T>C	p.V1105A														
c.3320T>C	p.L1107P									92	NT	NT			
c.3323delA	p.Y1108Sfs									12	NT	NT			
c.3331C>G	p.P1111A			103	88	NT	NT	NT	NT						
c.3342G>C	p.Q1114H									98	NT	NT	92	NT	NT
c.3356T>C	p.L1119P			94	104	NT	NT	NT	NT	98	NT	NT	84	89	~68
c.3362delG	p.G1121Vfs									12	NT	NT			
c.3367G>A	p.V1123M			76	103	NT	NT	NT	NT						
c.3404G>A	p.G1135E									98	NT	NT			
c.3418T>G	p.W1140G*									98	NT	NT	64	66	~35
c.3428T>A	p.L1143H			70	98	NT	NT	NT	NT						
c.3428T>C	p.L1143P	~80								132	NT	NT	109	NT	NT
c.3433G>C	p.G1145R									104	NT	NT			
c.3520G>A	p.G1147R									96	NT	NT			
c.3449T>G	p.L1150R									150	NT	NT			
c.3476G>T	p.W1159L			91	108	NT	NT	NT	NT						

(Continued)

TABLE 2 | Continued

Study	Protein change	Park et al., 2014				Foo et al., 2017				Boonen et al., 2019				Wiltshire et al., 2019				Rodrigue et al., 2019			
		DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	DR-GFP (%)	PARPi (%)	Cisplatin (%)	G2 > M (%)	RAD51 (%)	LMNA (%)	DR-GFP (%)	RAD51 (%)	LMNA (%)	LMNA (%)	PARPi (%)	RAD51 (%)	LMNA (%)	LMNA (%)		
	c.3478T>C			92	103	NT	NT	NT	NT	NT	100	NT	NT	NT							
	p.S1160P			81	96	NT	NT	NT	NT	NT	98	NT	NT	NT							
	c.3492G>T																				
	p.W1164C																				
	c.3494C>T																				
	p.S1165L																				
	c.3497delG																				
	p.G1166Vfs																				
	c.3500C>T																				
	p.T1167I																				
	c.3508C>T																				
	p.H1170Y																				
	c.3515T>C			13	17	NT	NT	NT	NT	NT											
	p.L1172P*			91	103	NT	NT	NT	NT	NT											
	c.3520G>A			82	99	NT	NT	NT	NT	NT											
	p.G1174R																				
	c.3539T>C			71	109	NT	NT	NT	NT	NT	72	NT	NT	NT	106	NT	NT	NT	NT		
	p.I1180T																				
	c.3548A>G			11	10	NT	NT	NT	NT	NT	48	NT	NT	NT							
	p.Y1183C																				
	c.3549C>G																				
	p.Y1183X																				

Nucleotide numbering reflects Human Genome Variation Society (HGVS) nomenclature where cDNA numbering +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, G2/M checkpoint assays and LMNA HR are shown. Only data taken from bar graphs 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.'

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 (Rodrigue et al., 2019). 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 (Foo et al., 2017). 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 (Ducy et al., 2019) 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 (Boonen et al., 2019), 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 human PALB2 cDNA at a safe-harbor locus in Palb2 knockout (KO) mouse embryonic stem (mES) cells and its subsequent expression from a relatively weak promoter (Boonen et al., 2019). Such a strategy avoids differences in PALB2 expression following siRNA-mediated knockdown and reduces possible artifacts 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 compared 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) (Boonen et al., 2019).

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 (Sy et al., 2009b; Zhang et al., 2009a,b; Jensen et al., 2010). Variants that affect PALB2's interaction capability with BRCA1 or BRCA2 are therefore predicted to impact HR. Here we first discuss the implications of variants in PALB2's CC domain (amino acids 9–44) (**Figure 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 (Sy et al., 2009b), or p.L21 or p.L24 by a proline (Zhang et al., 2009a), 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 (Foo et al., 2017). This variant was taken along by the three recent studies which all confirmed these findings (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019). A similar defect in HR was observed for p.L24S, which was also attributable to an impairment in the interaction with BRCA1 (Boonen et al., 2019; Wiltshire et al., 2019). 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 (Wiltshire et al., 2019). 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) (Foo et al., 2017; Boonen et al., 2019; Wiltshire et al., 2019). 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 toward the formation of PALB2 oligomers (Sy et al., 2009c; Buisson and Masson, 2012). Such complexes may shield PALB2 from ubiquitination-dependent degradation (Orthwein et al., 2015), leading to higher protein levels.

The consistency between the different studies (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019) was challenged by the analysis of p.R37H, which has previously been shown to represent a variant whose expression only moderately impacts protein function (Foo et al., 2017). p.R37H was shown to reduce HR only by ~20% (Foo et al., 2017; Wiltshire et al., 2019). Accordingly, the analysis by Foo et al. (2017) showed that p.R37H did not affect the interaction with BRCA1. In contrast, Rodrigue et al. (2019) 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. 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*) (Boonen et al., 2019), we observed a partial loss of the PALB2-BRCA1 interaction in co-immunoprecipitation experiments, as well as the recruitment of PALB2 to sites of DNA damage induced by laser micro-irradiation (Boonen et al., 2019). 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 853–1186) (**Figure 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) (Boonen et al., 2019). 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 (Boonen et al., 2019), suggesting that the low abundance of PALB2 protein is likely the result of protein instability. In contrast, Rodrigue et al. (2019) performed PALB2-BRCA2 co-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. 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 (Wiltshire et al., 2019). 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*) (Boonen et al., 2019). These results are in line with the fact that this variant impairs the PALB2-BRCA2 interaction, as shown previously by several studies (Oliver et al., 2009; Rodrigue et al., 2019; Wiltshire et al., 2019).

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 (Park et al., 2014; Boonen et al., 2019; Wiltshire et al., 2019). 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 et al. (2019) 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 micro-irradiation induced DNA damage, an effect that was also observed for p.Y28C and p.L35P. 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 signaled 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 the three recent studies (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019). 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. Differences therein may explain certain discrepancies in the outcomes, which we discuss below on the basis of several variants that were functionally characterized.

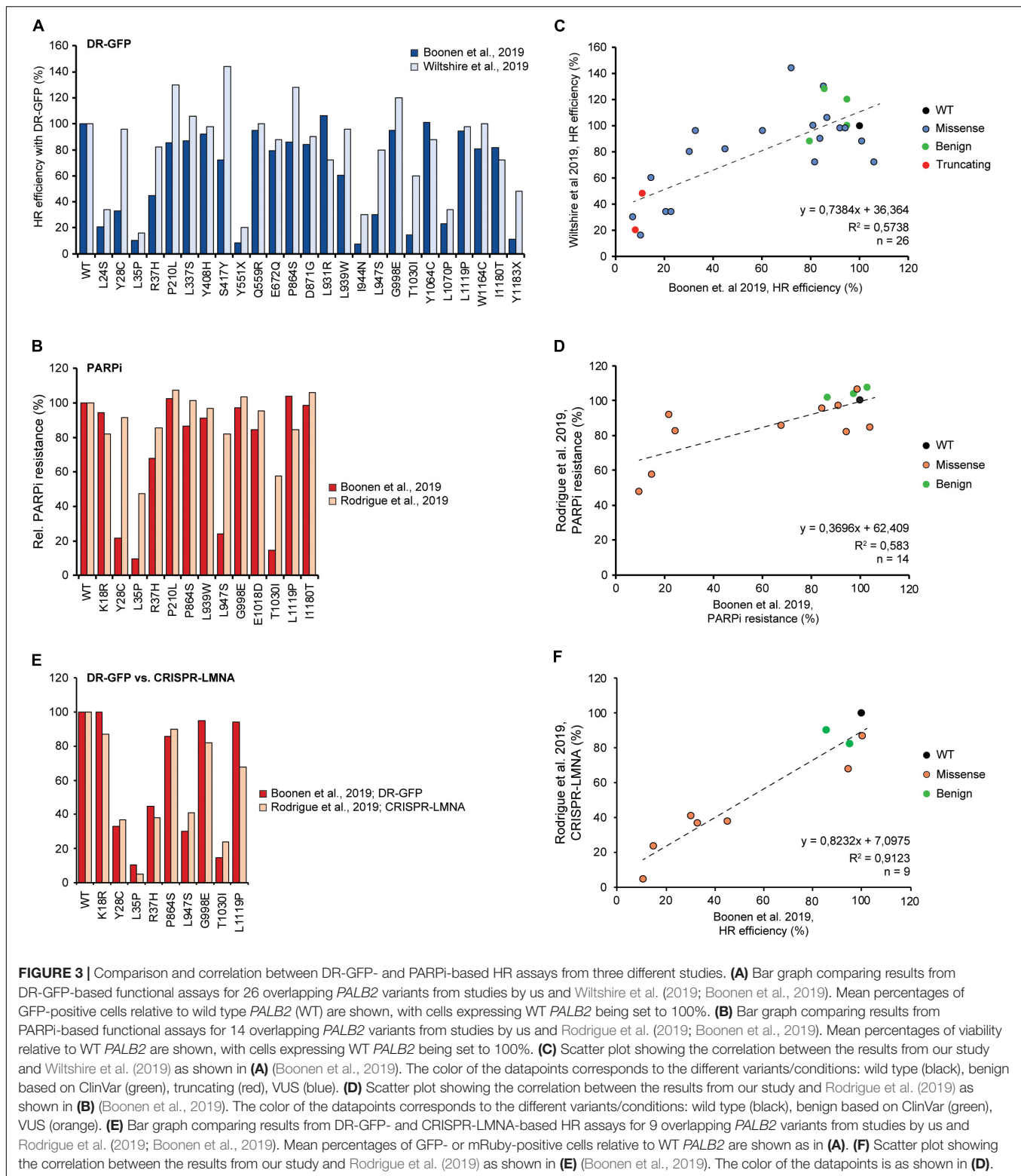
Overexpression of the *PALB2* cDNA may underestimate the functional effect of some variants. For instance, the FA-associated p.Y1183X *PALB2* variant, which is located three amino acids from the end of the protein, can lead to the expression of a near full-length *PALB2* protein. Stable expression of 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%) (Boonen et al., 2019). 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 (Wiltshire et al., 2019). Accordingly, Rodrigue et al. (2019) 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* (Rodrigue et al., 2019). 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 ($n = 26$) (Boonen et al., 2019; Wiltshire et al., 2019) and PARPi sensitivity assays ($n = 14$) (Boonen et al., 2019; Rodrigue et al., 2019), functional defects are almost always smaller when assessed by transient overexpression compared to stable integration and expression (Figures 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 ($R^2 = \sim 0.58$) between results from assays with transient overexpression versus

stable integration and expression (Figures 3C,D). However, this hypothesis is contradicted by the very good correlation ($R^2 = \sim 0.91$) (Figures 3E,F) between the effects of overlapping variants in DR-GFP and CRISPR-LMNA HR assays ($n = 9$) (Boonen et al., 2019; Rodrigue et al., 2019), 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 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 wild type *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 wild type *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 adaptations in order to survive. It is possible that such adaptations can influence the functional readout that is used.

As all three recent studies employed a cDNA-based complementation approach (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019), 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 (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019), 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 cells expressing mouse *Palb2*) (Boonen et al., 2019). Although it cannot be excluded that 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 (Boonen et al., 2019).

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 (Cotta-Ramusino et al., 2011; Menzel et al., 2011; Simhadri et al., 2019). 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* (Figure 2) (Boonen et al., 2019). 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* (Ceccaldi et al., 2015). 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 (Ceccaldi et al., 2015). 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 (Ma et al., 2012), 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 (Ma et al., 2012). As *PALB2* bears a highly conserved ETGE-type *KEAP1*-binding motif (amino acids 88–94), 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 (Xia et al., 2007), still interacts with *NRF2* as corroborated by Ma et al. (2012), 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 (Xia et al., 2007). 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 (Schlachter et al., 2011, 2012; Chaudhuri et al., 2016; Sidorova, 2017). 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 (Daza-Martin et al., 2019). 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 (Schlachter et al., 2011). *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 (Petermann et al., 2010; Zellweger et al., 2015; Godin et al., 2016). 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 (Murphy et al., 2014). 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 (Nikkila et al., 2013). 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* (Schlachter et al., 2011; Daza-Martin et al., 2019). 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 the 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 (Lek et al., 2016; Karczewski et al., 2019), a variety of *in silico* tools, which are both publicly and commercially available, can aid in

the interpretation of VUS in clinical diagnostic settings (Richards et al., 2015). 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 (Grimm et al., 2015; Miosge et al., 2015). 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 (Sun et al., 2016). 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 (Sun et al., 2016). This high rate of false predictions is in agreement with recent data from us and Rodrigue et al. (2019), showing that *in silico* prediction tools all strongly overpredicted the percentage of deleterious variants in *PALB2* (Boonen et al., 2019). Consistently, these studies observed a poor correlation between results from DR-GFP assays and predictions by CADD ($R^2 = 0.08$) or REVEL ($R^2 = 0.11$) (Boonen et al., 2019), and between results from PARPi sensitivity assays and M-CAP ($R^2 = 0.33$), VEST ($R^2 = 0.07$) or REVEL ($R^2 = 0.27$) (Rodrigue et al., 2019). Due to this lack of consistency and poor performance, computational predictions are not considered strong evidence for or against pathogenicity (Richards et al., 2015). 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. (2019), the measured HR efficiency for 248 *BRCA1* and 207 *BRCA2* variants was used to recalibrate 40 *in silico* algorithms. Optimized thresholds based on such functional data significantly improved the accuracy of many of these algorithms. However, optimized algorithms for one gene may perform poorly when applied to another gene. This is perhaps not surprising as each functional domain may harbor 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 (Priestley et al., 2019), 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) (Lek et al., 2016; Karczewski et al., 2019) and 99% of these variants are rare

with a minor allele frequency of <0.005 (Starita et al., 2017). 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 (Sun et al., 2016; Boonen et al., 2019). Thus, the accelerated rate of VUS discovery makes a one-at-a-time approach, or even semi high-throughput methods, 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 (Green et al., 2013; Kalia et al., 2017), 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 (Findlay et al., 2018). 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* (Starita et al., 2018). We expect that in the near future such assays will be extended to analyzing variants in genes such as *PALB2*, 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* (Boonen et al., 2019), 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 (Yue et al., 2005; Redler et al., 2016; Matreyek et al., 2018). Therefore, high-throughput assessment of *PALB2* variant protein abundance, by employing techniques such as VAMP-seq (Matreyek et al., 2018) or Stable-seq (Kim et al., 2013), 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.

TOWARD 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 (Park et al., 2014; Foo et al., 2017; Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019), 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 (Richards et al., 2015), 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 (Gaildrat et al., 2010). 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 (Lopez-Perolio et al., 2019). 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 (Byrd et al., 2016). 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* (Kuznetsov et al., 2008; Chang et al., 2009; Mesman et al., 2018, 2020) and would allow for the introduction and functional analysis of splice variants in coding and non-coding regions, further improving their classification.

TOWARD 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) (Shimelis et al., 2017; Mesman et al., 2019). With regard to *PALB2*, truncating variants have been associated with an odds ratio of 7.46 (95% CI, 5.12–11.19) (Couch et al., 2017), whereas the frequently occurring p.L939W missense variant has been associated with an odds ratio of 1.05 (95% CI, 0.83–1.32) (Southey et al., 2016), which is in agreement with recent data from Rodrigue et al. (2019) and Wiltshire et al. (2019) showing that this variant does not impact the HR efficiency (~4% reduction in HR when compared to WT). In contrast, results from us and Park et al. (2014) showed that this variant did impair HR to some degree (40 and 15% reduction in HR when compared to WT, respectively) (Boonen et al., 2019). 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 (Schlachter et al., 2011; Murphy et al., 2014; Chaudhuri et al., 2016; Daza-Martin et al., 2019), 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 (Schlachter et al., 2011; Daza-Martin et al., 2019). 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 reliant on alternative DNA repair mechanisms to survive and proliferate. Therefore, conventional treatment strategies (especially for HR-deficient tumors) 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 (Bryant et al., 2005; Farmer et al., 2005), a therapeutic strategy that has emerged for

BRCA1- and *BRCA2*-mutated breast and ovarian tumors (Audeh et al., 2010; Tutt et al., 2010; Lord and Ashworth, 2017). Consequently, it is of great importance to identify deleterious *PALB2* VUS that lead to HR deficiency and for which corresponding tumors may similarly respond to PARPi-based therapy.

With regard to the studies that functionally analyzed VUS in *PALB2* (Park et al., 2014; Foo et al., 2017; Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2019), it is clear that within each study, the HR efficiency correlated extremely well with PARPi sensitivity, exhibiting a strong positive correlation in mES cells ($R^2 = 0.804$) (Boonen et al., 2019) and human cell lines ($R^2 = 0.68$) (Rodrigue et al., 2019). Similar results were obtained for sensitivity assays with cisplatin ($R^2 = 0.8313$) (Boonen et al., 2019; Wiltshire et al., 2019), a commonly-used chemotherapeutic for many cancers, including breast and ovarian cancer. Similar to that in many *BRCA1*- and *BRCA2*-associated tumors (Maxwell et al., 2017; Riaz et al., 2017), 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) (Lee et al., 2018; Li et al., 2019). Such *PALB2*-null cancers all exhibited HR deficiency, with some tumors even showing HR deficiency while the wild type allele was retained (Lee et al., 2018; Li et al., 2019), 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 (Plon et al., 2008).

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 (Figure 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 (Richards et al., 2015; Brnich et al., 2019; Monteiro et al., 2020). 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*.

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RB, MV, and HA conducted the literature research and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

- Antoniou, A., Pharoah, P. D., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., et al. (2003). Average risks of breast and ovarian cancer associated with *BRCA1* or *BRCA2* mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am. J. Hum. Genet.* 72, 1117–1130.
- Antoniou, A. C., Casadei, S., Heikkinen, T., Barrowdale, D., Pylkas, K., Roberts, J., et al. (2014). Breast-cancer risk in families with mutations in *PALB2*. *N. Engl. J. Med.* 371, 497–506.
- Audeh, M. W., Carmichael, J., Penson, R. T., Friedlander, M., Powell, B., Bell-McGuinn, K. M., et al. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with *BRCA1* or *BRCA2* mutations and recurrent ovarian

- cancer: a proof-of-concept trial. *Lancet* 376, 245–251. doi: 10.1016/s0140-6736(10)60893-8
- Bignell, G., Micklem, G., Stratton, M. R., Ashworth, A., and Wooster, R. (1997). The BRCA repeats are conserved in mammalian BRCA2 proteins. *Hum. Mol. Genet.* 6, 53–58. doi: 10.1093/hmg/6.1.53
- Bleuyard, J. Y., Buisson, R., Masson, J. Y., and Esashi, F. (2012). ChAM, a novel motif that mediates PALB2 intrinsic chromatin binding and facilitates DNA repair. *EMBO Rep.* 13, 135–141. doi: 10.1038/embor.2011.243
- Boonen, R., Rodrigue, A., Stoepker, C., Wiegand, W. W., Vroliing, B., Sharma, M., et al. (2019). Functional analysis of genetic variants in the high-risk breast cancer susceptibility gene PALB2. *Nat. Commun.* 10:5296.
- Bouwman, P., van der Gulden, H., van der Heijden, I., Drost, R., Klijn, C. N., Prasetyanti, P., et al. (2013). A high-throughput functional complementation assay for classification of BRCA1 missense variants. *Cancer Discov.* 3, 1142–1155. doi: 10.1158/2159-8290.cd-13-0094
- Brnich, S. E., Abou Tayoun, A. N., Couch, F. J., Cutting, G. R., Greenblatt, M. S., Heinen, C. D., et al. (2019). Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework. *Genome Med.* 12:3.
- Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., et al. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913–917. doi: 10.1038/nature03443
- Buisson, R., and Masson, J. Y. (2012). PALB2 self-interaction controls homologous recombination. *Nucleic Acids Res.* 40, 10312–10323. doi: 10.1093/nar/gks807
- Byrd, P. J., Stewart, G. S., Smith, A., Eaton, C., Taylor, A. J., Guy, C., et al. (2016). A hypomorphic PALB2 allele gives rise to an unusual form of FA-N associated with lymphoid tumour development. *PLoS Genet.* 12:e1005945. doi: 10.1371/journal.pgen.1005945
- Callen, E., Zong, D., Wu, W., Wong, N., Stanlie, A., Ishikawa, M., et al. (2020). 53BP1 enforces distinct pre- and post-resection blocks on homologous recombination. *Mol Cell* 77:e27.
- Ceccaldi, R., Liu, J. C., Amunugama, R., Hajdu, I., Primack, B., Petalcorin, M. I., et al. (2015). Homologous-recombination-deficient tumours are dependent on Poltheta-mediated repair. *Nature* 518, 258–262. doi: 10.1038/nature14184
- Chang, S., Biswas, K., Martin, B. K., Stauffer, S., and Sharan, S. K. (2009). Expression of human BRCA1 variants in mouse ES cells allows functional analysis of BRCA1 mutations. *J. Clin. Invest.* 119, 3160–3171. doi: 10.1172/jci39836
- Chapman, J. R., Taylor, M. R., and Boulton, S. J. (2012). Playing the end game: DNA double-strand break repair pathway choice. *Mol. Cell* 47, 497–510. doi: 10.1016/j.molcel.2012.07.029
- Chaudhuri, A. R., Callen, E., Ding, X., Gogola, E., Duarte, A. A., Lee, J. E., et al. (2016). Erratum: replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* 539:456. doi: 10.1038/nature19826
- Ciccia, A., and Elledge, S. J. (2010). The DNA damage response: making it safe to play with knives. *Mol. Cell* 40, 179–204. doi: 10.1016/j.molcel.2010.09.019
- Cotta-Ramusino, C., McDonald, E. R. III, Hurov, K., Sowa, M. E., Harper, J. W., and Elledge, S. J. (2011). A DNA damage response screen identifies RHINO, a 9-1-1 and TopBP1 interacting protein required for ATR signaling. *Science* 332, 1313–1317. doi: 10.1126/science.1203430
- Couch, F. J., Shimelis, H., Hu, C., Hart, S. N., Polley, E. C., Na, J., et al. (2017). Associations between cancer predisposition testing panel genes and breast cancer. *JAMA Oncol.* 3, 1190–1196.
- Daza-Martin, M., Starowicz, K., Jamshad, M., Tye, S., Ronson, G. E., MacKay, H. L., et al. (2019). Isomerization of BRCA1-BARD1 promotes replication fork protection. *Nature* 571, 521–527. doi: 10.1038/s41586-019-1363-4
- Deans, A. J., and West, S. C. (2011). DNA interstrand crosslink repair and cancer. *Nat. Rev. Cancer* 11, 467–480. doi: 10.1038/nrc3088
- Densham, R. M., Garvin, A. J., Stone, H. R., Strachan, J., Baldock, R. A., Daza-Martin, M., et al. (2016). Human BRCA1-BARD1 ubiquitin ligase activity counteracts chromatin barriers to DNA resection. *Nat. Struct. Mol. Biol.* 23, 647–655. doi: 10.1038/nsmb.3236
- Ducy, M., Sesma-Sanz, L., Guittion-Sert, L., Lashgari, A., Gao, Y., Brahiti, N., et al. (2019). The tumor suppressor PALB2: inside out. *Trends Biochem. Sci.* 44, 226–240. doi: 10.1016/j.tibs.2018.10.008
- Esashi, F., Christ, N., Gannon, J., Liu, Y., Hunt, T., Jasin, M., et al. (2005). CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* 434, 598–604. doi: 10.1038/nature03404
- Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N., Johnson, D. A., Richardson, T. B., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917–921. doi: 10.1038/nature03445
- Findlay, G. M., Daza, R. M., Martin, B., Zhang, M. D., Leith, A. P., Gasperini, M., et al. (2018). Accurate classification of BRCA1 variants with saturation genome editing. *Nature* 562, 217–222. doi: 10.1038/s41586-018-0461-z
- Foo, T. K., Tischkowitz, M., Simhadri, S., Boshari, T., Zayed, N., Burke, K. A., et al. (2017). Compromised BRCA1-PALB2 interaction is associated with breast cancer risk. *Oncogene* 36, 4161–4170. doi: 10.1038/ncr.2017.46
- Gaildrat, P., Killian, A., Martins, A., Tournier, I., Frebourg, T., and Tosi, M. (2010). Use of splicing reporter minigene assay to evaluate the effect on splicing of unclassified genetic variants. *Methods Mol. Biol.* 653, 249–257. doi: 10.1007/978-1-60761-759-4_15
- Godin, S. K., Sullivan, M. R., and Bernstein, K. A. (2016). Novel insights into RAD51 activity and regulation during homologous recombination and DNA replication. *Biochem. Cell Biol.* 94, 407–418. doi: 10.1139/bcb-2016-0012
- Green, R. C., Berg, J. S., Grody, W. W., Kalia, S. S., Korf, B. R., Martin, C. L., et al. (2013). ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. *Genet. Med.* 15, 565–574. doi: 10.1038/gim.2013.73
- Grimm, D. G., Azencott, C. A., Aichele, F., Gieraths, U., MacArthur, D. G., Samocha, K. E., et al. (2015). The evaluation of tools used to predict the impact of missense variants is hindered by two types of circularity. *Hum. Mutat.* 36, 513–523. doi: 10.1002/humu.22768
- Hart, S. N., Hoskin, T., Shimelis, H., Moore, R. M., Feng, B., Thomas, A., et al. (2019). Comprehensive annotation of BRCA1 and BRCA2 missense variants by functionally validated sequence-based computational prediction models. *Genet. Med.* 21, 71–80. doi: 10.1038/s41436-018-0018-4
- Hofstatter, E. W., Domchek, S. M., Miron, A., Garber, J., Wang, M., Compositeschi, K., et al. (2011). PALB2 mutations in familial breast and pancreatic cancer. *Fam. Cancer* 10, 225–231. doi: 10.1007/s10689-011-9426-1
- Howlett, N. G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., et al. (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 297, 606–609. doi: 10.1126/science.1073834
- Hu, C., LaDuca, H., Shimelis, H., Polley, E. C., Lilyquist, J., Hart, S. N., et al. (2018). Multigene hereditary cancer panels reveal high-risk pancreatic cancer susceptibility genes. *JCO Precis. Oncol.* 2:291.
- Hustedt, N., and Durocher, D. (2016). The control of DNA repair by the cell cycle. *Nat. Cell Biol.* 19, 1–9.
- Jensen, R. B., Carreira, A., and Kowalczykowski, S. C. (2010). Purified human BRCA2 stimulates RAD51-mediated recombination. *Nature* 467, 678–683. doi: 10.1038/nature09399
- Jones, S., Hruban, R. H., Kamiyama, M., Borges, M., Zhang, X., Parsons, D. W., et al. (2009). Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* 324:217. doi: 10.1126/science.1171202
- Kalia, S. S., Adelman, K., Bale, S. J., Chung, W. K., Eng, C., Evans, J. P., et al. (2017). Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. *Genet. Med.* 19, 249–255. doi: 10.1038/gim.2016.190
- Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alföldi, J., Wang, Q., et al. (2019). Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. *bioRxiv* [Preprint] doi: 10.1101/531210
- Kass, E. M., Helgadottir, H. R., Chen, C. C., Barbera, M., Wang, R., Westermark, U. K., et al. (2013). Double-strand break repair by homologous recombination in primary mouse somatic cells requires BRCA1 but not the ATM kinase. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5564–5569. doi: 10.1073/pnas.1216824110
- Kim, I., Miller, C. R., Young, D. L., and Fields, S. (2013). High-throughput analysis of in vivo protein stability. *Mol. Cell Proteomics* 12, 3370–3378.
- Kuznetsov, S. G., Liu, P., and Sharan, S. K. (2008). Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat. Med.* 14, 875–881. doi: 10.1038/nm.1719
- Landrum, M. J., Lee, J. M., Riley, G. R., Jang, W., Rubinstein, W. S., Church, D. M., et al. (2014). ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res.* 42, D980–D985.

- Lee, J. E. A., Li, N., Rowley, S. M., Cheasley, D., Zethoven, M., McInerney, S., et al. (2018). Molecular analysis of PALB2-associated breast cancers. *J. Pathol.* 245, 53–60. doi: 10.1002/path.5055
- Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., et al. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 536, 285–291.
- Li, A., Geyer, F. C., Blecua, P., Lee, J. Y., Selenica, P., Brown, D. N., et al. (2019). Homologous recombination DNA repair defects in PALB2-associated breast cancers. *NPJ Breast Cancer* 5:23.
- Lopez-Perolio, I., Leman, R., Behar, R., Lattimore, V., Pearson, J. F., Castera, L., et al. (2019). Alternative splicing and ACMG-AMP-2015-based classification of PALB2 genetic variants: an ENIGMA report. *J. Med. Genet.* 56, 453–460. doi: 10.1136/jmedgenet-2018-105834
- Lord, C. J., and Ashworth, A. (2016). BRCAness revisited. *Nat. Rev. Cancer* 16, 110–120. doi: 10.1038/nrc.2015.21
- Lord, C. J., and Ashworth, A. (2017). PARP inhibitors: synthetic lethality in the clinic. *Science* 355, 1152–1158. doi: 10.1126/science.aam7344
- Luijsterburg, M. S., Typas, D., Caron, M. C., Wiegant, W. W., van den Heuvel, D., Boonen, R. A., et al. (2017). A PALB2-interacting domain in RNF168 couples homologous recombination to DNA break-induced chromatin ubiquitylation. *eLife* 6:e20922.
- Ma, J., Cai, H., Wu, T., Sobhian, B., Huo, Y., Alcivar, A., et al. (2012). PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function. *Mol. Cell Biol.* 32, 1506–1517. doi: 10.1128/mcb.06271-11
- Marini, F., Rawal, C. C., Liberi, G., and Pellicoli, A. (2019). Regulation of DNA double strand breaks processing: focus on barriers. *Front. Mol. Biosci.* 6:55. doi: 10.3389/fmolb.2019.00055
- Matreyek, K. A., Starita, L. M., Stephany, J. J., Martin, B., Chiasson, M. A., Gray, V. E., et al. (2018). Multiplex assessment of protein variant abundance by massively parallel sequencing. *Nat. Genet.* 50, 874–882. doi: 10.1038/s41588-018-0122-z
- Maxwell, K. N., Wubbenhorst, B., Wenz, B. M., De Sloover, D., Pluta, J., Emery, L., et al. (2017). BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. *Nat. Commun.* 8:319.
- McCabe, N., Turner, N. C., Lord, C. J., Kluzek, K., Bialkowska, A., Swift, S., et al. (2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res.* 66, 8109–8115. doi: 10.1158/0008-5472.can-06-0140
- Menzel, T., Nahse-Kumpf, V., Kousholt, A. N., Klein, D. K., Lund-Andersen, C., Lees, M., et al. (2011). A genetic screen identifies BRCA2 and PALB2 as key regulators of G2 checkpoint maintenance. *EMBO Rep.* 12, 705–712. doi: 10.1038/embor.2011.99
- Mesman, R. L. S., Calleja, F., Hendriks, G., Morolli, B., Misovic, B., Devilee, P., et al. (2018). The functional impact of variants of uncertain significance in BRCA2. *Genet. Med.* 21, 293–302.
- Mesman, R. L. S., Calleja, F., Hendriks, G., Morolli, B., Misovic, B., Devilee, P., et al. (2019). The functional impact of variants of uncertain significance in BRCA2. *Genet. Med.* 21, 293–302. doi: 10.1038/s41436-018-0052-2
- Mesman, R. L. S., Calléja, F. M. G. R., de la Hoya, M., Devilee, P., van Asperen, C. J., Vrieling, H., et al. (2020). Alternative mRNA splicing can attenuate the pathogenicity of presumed loss-of-function variants in BRCA2. *Genet. Med.* doi: 10.1038/s41436-020-0814-5.
- Miosge, L. A., Field, M. A., Sontani, Y., Cho, V., Johnson, S., Palkova, A., et al. (2015). Comparison of predicted and actual consequences of missense mutations. *Proc. Natl. Acad. Sci. U.S.A.* 112, E5189–E5198.
- Monteiro, A. N., Bouwman, P., Kousholt, A. N., Eccles, D. M., Millot, G. A., Masson, J. Y., et al. (2020). Variants of uncertain clinical significance in hereditary breast and ovarian cancer genes: best practices in functional analysis for clinical annotation. *J. Med. Genet.* doi: 10.1136/jmedgenet-2019-106368
- Murai, J., Huang, S. Y., Das, B. B., Renaud, A., Zhang, Y., Doroshov, J. H., et al. (2012). Trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Res.* 72, 5588–5599. doi: 10.1158/0008-5472.can-12-2753
- Murphy, A. K., Fitzgerald, M., Ro, T., Kim, J. H., Rabinowitsch, A. I., Chowdhury, D., et al. (2014). Phosphorylated RPA recruits PALB2 to stalled DNA replication forks to facilitate fork recovery. *J. Cell Biol.* 206, 493–507. doi: 10.1083/jcb.201404111
- Nguyen, L., Martens, J., Van Hoeck, A., and Cuppen, E. (2020). Pan-cancer landscape of homologous recombination deficiency. *bioRxiv[Preprint]* doi: 10.1101/2020.01.13.905026
- Nikkila, J., Parpys, A. C., Pylkas, K., Bose, M., Huo, Y., Borgmann, K., et al. (2013). Heterozygous mutations in PALB2 cause DNA replication and damage response defects. *Nat. Commun.* 4:2578.
- Nik-Zainal, S., Davies, H., Staaf, J., Ramakrishna, M., Glodzik, D., Zou, X., et al. (2016). Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature* 534, 47–54.
- Oliver, A. W., Swift, S., Lord, C. J., Ashworth, A., and Pearl, L. H. (2009). Structural basis for recruitment of BRCA2 by PALB2. *EMBO Rep.* 10, 990–996. doi: 10.1038/embor.2009.126
- Orthwein, A., Noordermeer, S. M., Wilson, M. D., Landry, S., Enchev, R. I., Sherker, A., et al. (2015). A mechanism for the suppression of homologous recombination in G1 cells. *Nature* 528, 422–426. doi: 10.1038/nature16142
- Park, J. Y., Singh, T. R., Nassar, N., Zhang, F., Freund, M., Hanenberg, H., et al. (2014). Breast cancer-associated missense mutants of the PALB2 WD40 domain, which directly binds RAD51C, RAD51 and BRCA2, disrupt DNA repair. *Oncogene* 33, 4803–4812. doi: 10.1038/onc.2013.421
- Petermann, E., Orta, M. L., Issaeva, N., Schultz, N., and Helleday, T. (2010). Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol. Cell* 37, 492–502. doi: 10.1016/j.molcel.2010.01.021
- Plon, S. E., Eccles, D. M., Easton, D., Foulkes, W. D., Genuardi, M., Greenblatt, M. S., et al. (2008). Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum. Mutat.* 29, 1282–1291. doi: 10.1002/humu.20880
- Polak, P., Kim, J., Braunstein, L. Z., Karlic, R., Haradhavala, N. J., Tiao, G., et al. (2017). A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer. *Nat. Genet.* 49, 1476–1486. doi: 10.1038/ng.3934
- Prakash, R., Zhang, Y., Feng, W., and Jasin, M. (2015). Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb. Perspect. Biol.* 7:a016600. doi: 10.1101/cshperspect.a016600
- Priestley, P., Baber, J., Lolkema, M. P., Steeghs, N., de Bruijn, E., Shale, C., et al. (2019). Pan-cancer whole-genome analyses of metastatic solid tumours. *Nature* 575, 210–216. doi: 10.1038/s41586-019-1689-y
- Redler, R. L., Das, J., Diaz, J. R., and Dokholyan, N. V. (2016). Protein destabilization as a common factor in diverse inherited disorders. *J. Mol. Evol.* 82, 11–16. doi: 10.1007/s00239-015-9717-5
- Riaz, N., Blecua, P., Lim, R. S., Shen, R., Higginson, D. S., Weinhold, N., et al. (2017). Pan-cancer analysis of bi-allelic alterations in homologous recombination DNA repair genes. *Nat. Commun.* 8:857.
- Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for molecular pathology. *Genet. Med.* 17, 405–424.
- Rodrigue, A., Margailan, G., Torres Gomes, T., Coulombe, Y., Montalban, G., da Costa, E. S. C. S., et al. (2019). A global functional analysis of missense mutations reveals two major hotspots in the PALB2 tumor suppressor. *Nucleic Acids Res.* 47, 10662–10677. doi: 10.1093/nar/gkz780
- Sawyer, S. L., Tian, L., Kahkonen, M., Schwartzentruber, J., Kircher, M., Mendelian, G., et al. (2015). Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype. *Cancer Discov.* 5, 135–142. doi: 10.1158/2159-8290.cd-14-1156
- Schlacher, K., Christ, N., Siaud, N., Egashira, A., Wu, H., and Jasin, M. (2011). Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell* 145, 529–542. doi: 10.1016/j.cell.2011.03.041
- Schlacher, K., Wu, H., and Jasin, M. (2012). A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer Cell* 22, 106–116. doi: 10.1016/j.ccr.2012.05.015
- Shimelis, H., Mesman, R. L. S., Von Nicolai, C., Ehlen, A., Guidugli, L., Martin, C., et al. (2017). BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. *Cancer Res.* 77, 2789–2799.

- Sidorova, J. (2017). A game of substrates: replication fork remodeling and its roles in genome stability and chemo-resistance. *Cell Stress* 1, 115–133. doi: 10.15698/cst2017.12.114
- Simhadri, S., Vincelli, G., Huo, Y., Misenko, S., Foo, T. K., Ahlskog, J., et al. (2019). PALB2 connects BRCA1 and BRCA2 in the G2/M checkpoint response. *Oncogene* 38, 1585–1596. doi: 10.1038/s41388-018-0535-2
- Sishc, B. J., and Davis, A. J. (2017). The role of the core non-homologous end joining factors in carcinogenesis and cancer. *Cancers (Basel)* 9:81. doi: 10.3390/cancers9070081
- Slater, E. P., Langer, P., Niemczyk, E., Strauch, K., Butler, J., Habbe, N., et al. (2010). PALB2 mutations in European familial pancreatic cancer families. *Clin. Genet* 78, 490–494. doi: 10.1111/j.1399-0004.2010.01425.x
- Southey, M. C., Goldgar, D. E., Winqvist, R., Pylkas, K., Couch, F., Tischkowitz, M., et al. (2016). PALB2, CHEK2 and ATM rare variants and cancer risk: data from COGS. *J. Med. Genet.* 53, 800–811.
- Starita, L. M., Ahituv, N., Dunham, M. J., Kitzman, J. O., Roth, F. P., Seelig, G., et al. (2017). Variant interpretation: functional assays to the rescue. *Am. J. Hum. Genet.* 101, 315–325. doi: 10.1016/j.ajhg.2017.07.014
- Starita, L. M., Islam, M. M., Banerjee, T., Adamovich, A. I., Gullingsrud, J., Fields, S., et al. (2018). A multiplex homology-directed DNA repair assay reveals the impact of more than 1,000 BRCA1 missense substitution variants on protein function. *Am. J. Hum. Genet.* 103, 498–508. doi: 10.1016/j.ajhg.2018.07.016
- Sun, S., Yang, F., Tan, G., Costanzo, M., Oughtred, R., Hirschman, J., et al. (2016). An extended set of yeast-based functional assays accurately identifies human disease mutations. *Genome Res.* 26, 670–680. doi: 10.1101/gr.192526.115
- Sy, S. M., Huen, M. S., and Chen, J. (2009a). MRG15 is a novel PALB2-interacting factor involved in homologous recombination. *J. Biol. Chem.* 284, 21127–21131. doi: 10.1074/jbc.c109.023937
- Sy, S. M., Huen, M. S., and Chen, J. (2009b). PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7155–7160. doi: 10.1073/pnas.0811159106
- Sy, S. M., Huen, M. S., Zhu, Y., and Chen, J. (2009c). PALB2 regulates recombinational repair through chromatin association and oligomerization. *J. Biol. Chem.* 284, 18302–18310. doi: 10.1074/jbc.m109.016717
- Symington, L. S. (2016). Mechanism and regulation of DNA end resection in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.* 51, 195–212. doi: 10.1016/j.crb.2016.11.025
- Tischkowitz, M., and Xia, B. (2010). PALB2/FANCN: recombining cancer and Fanconi anemia. *Cancer Res.* 70, 7353–7359. doi: 10.1158/0008-5472.can-10-1012
- Tischkowitz, M., Xia, B., Sabbaghian, N., Reis-Filho, J. S., Hamel, N., Li, G., et al. (2007). Analysis of PALB2/FANCN-associated breast cancer families. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6788–6793. doi: 10.1073/pnas.0701724104
- Tutt, A., Robson, M., Garber, J. E., Domchek, S. M., Audeh, M. W., Weitzel, J. N., et al. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet* 376, 235–244. doi: 10.1016/s0140-6736(10)60892-6
- Wiltshire, T., Ducey, M., Foo, T. K., Hu, C., Lee, K. Y., Belur Nagaraj, A., et al. (2019). Functional characterization of 84 PALB2 variants of uncertain significance. *Genet. Med.* 22, 622–632. doi: 10.1038/s41436-019-0682-z
- Wong, A. K., Pero, R., Ormonde, P. A., Tavtigian, S. V., and Bartel, P. L. (1997). RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J. Biol. Chem.* 272, 31941–31944. doi: 10.1074/jbc.272.51.31941
- Woods, N. T., Baskin, R., Golubeva, V., Jhuraney, A., De-Gregoriis, G., Vaclova, T., et al. (2016). Functional assays provide a robust tool for the clinical annotation of genetic variants of uncertain significance. *NPJ Genom. Med.* 1:16001.
- Xia, B., Dorsman, J. C., Ameziane, N., de Vries, Y., Rooimans, M. A., Sheng, Q., et al. (2007). Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. *Nat. Genet.* 39, 159–161. doi: 10.1038/ng1942
- Xia, B., Sheng, Q., Nakanishi, K., Ohashi, A., Wu, J., Christ, N., et al. (2006). Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol. Cell* 22, 719–729. doi: 10.1016/j.molcel.2006.05.022
- Yang, X., Leslie, G., Doroszuk, A., Schneider, S., Allen, J., Decker, B., et al. (2020). Cancer risks associated with germline PALB2 pathogenic variants: an international study of 524 families. *J. Clin. Oncol.* 38, 674–685.
- Yue, P., Li, Z., and Moulton, J. (2005). Loss of protein structure stability as a major causative factor in monogenic disease. *J. Mol. Biol.* 353, 459–473. doi: 10.1016/j.jmb.2005.08.020
- Zellweger, R., Dalcher, D., Mutreja, K., Berti, M., Schmid, J. A., Herrador, R., et al. (2015). Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *J. Cell Biol.* 208, 563–579. doi: 10.1083/jcb.201406099
- Zhang, F., Fan, Q., Ren, K., and Andreassen, P. R. (2009a). PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol. Cancer Res.* 7, 1110–1118. doi: 10.1158/1541-7786.mcr-09-0123
- Zhang, F., Ma, J., Wu, J., Ye, L., Cai, H., Xia, B., et al. (2009b). PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr. Biol.* 19, 524–529. doi: 10.1016/j.cub.2009.02.018
- Zong, D., Adam, S., Wang, Y., Sasanuma, H., Callen, E., Murga, M., et al. (2019). BRCA1 haploinsufficiency is masked by RNF168-mediated chromatin ubiquitylation. *Mol. Cell* 73, 1267–1281.e7. doi: 10.1016/j.molcel.2018.12.010

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.

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