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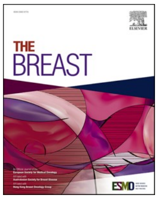

Large-scale meta-analysis and precision functional assays identify FANCM regions in which PTVs confer different risks for ER-negative and triple-negative breast cancer

Amandine Billaud[1,2,3,#], Gisella Figlioli[1,4,5,#], Clémence Mooser[6], Irene Casamassima[6,7], Violette Azzoni[6], Jahnavi Srivatsa[1], Mara Colombo[8], Laura Caleca[8], Thomas U. Ahearn[9], Irene L. Andrulis[10,11], Antonis C. Antoniou[12], Matthias W. Beckmann[13], Sabine Behrens[14], Marina Bermisheva[15], Natalia V. Bogdanova[16], Manjeet K. Bolla[12], Bernardo Bonanni[17], Thomas Brüning[18], Nicola J. Camp[19], Archie Campbell[20], Jose E. Castelao[21], Melissa H. Cessna[22], Jenny Chang-Claude[14,23], NBCS Collaborators[24,25,26,27,28,29,30,31,32,33,34,35], Kamila Czene[36], Joe Dennis[12], Peter Devilee[37,38], Thilo Dörk[39], Alison M. Dunning[40], Mikael Eriksson[36], D.Gareth Evans[41,42], Peter A. Fasching[13], Jonine D. Figueroa[9,43,44], Marike Gabrielson[36], Manuela Gago-Dominguez[45], Anna González-Neira[46,47], Pascal Guénel[48], Andreas Hadjisavvas[49], Eric Hahnen[50,51], Ute Hamann[52], Peter Hillemanns[39], Antoinette Hollestelle[53], Maartje J. Hooning[53], Reiner Hoppe[54,55], Anthony Howell[41,42], kConFab Investigators[56,57], Anna Jakubowska[58,59], Vessela N. Kristensen[25,35], Jan Lubiński[58], Michael Lush[12], Siranoush Manoukian[60], Dimitrios Mavroudis[61], Roger L. Milne[62,63,64], Anna Marie Mulligan[65,66], William G. Newman[41,42], Nadia Obi[67,68], Mihalis I. Panayiotidis[49,69], Guillermo Pita[46], Muhammad U. Rashid[52,70], Valerie Rhenius[40], Emmanouil Saloustros[71], Elinor J. Sawyer[72], Rita K. Schmutzler[50,51,73], Mitul Shah[40], Melissa C. Southey[62,64,74], Amanda B. Spurdle[75], Ian Tomlinson[76], Thérèse Truong[48], Qin Wang[12], Camilla Wendt[77,78], Paul L. Auer[79], Nicholas J. Boddicker[80], Clara Bodelon[81], Elizabeth S. Burnside[82], Fei Chen[83], Fergus J. Couch[84], Susan M. Domchek[85], Heather A. Eliassen[86], Christopher Haiman[83], James M. Hodge[81], Chunling Hu[84], Hongyan Huang[87], Sara Lindstrom[88], Maria Elena Martinez[89], Katherine L. Nathanson[85], Susan L. Neuhausen[90], Katie M. O'Brien[91], Janet E. Olson[92], Julie R. Palmer[93], Alpa V. Patel[81], Kathryn J. Ruddy[92], Dale P. Sandler[91], Lauren R. Teras[81], Clarice R. Weinberg[94], Jeffrey N. Weitzel[95], Stacey J. Winham[80], Siddhartha Yadav[96], Song Yao[97], Gary Zirpoli[93], Marketa Janatova[98], Zdenek Kleibl[98,99], Petra Kleiblova[98,100], Jana Soukupova[98], CZECANCA consortium[98], Qihong Zhao[101], Lisa Devereux[101,102], Paul A. James[102,103], Ian G. Campbell[101,102], Tu Nguyen-Dumont[74,104], James G. Dowty[63], Nadine Andrieu[105,106,107,108], Fabienne Lesueur[105,106,107,108], Dominique Stoppa-Lyonnet[109,110,111], GENESIS[105,106,107,108,109,110,111], Miguel de la Hoya[112],

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* Corresponding author
E-mail address: paolo.peterlongo@ieo.it (P. Peterlongo).

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Paolo Radice[8], Claus Storgaard Sørensen[6], Paolo Peterlongo[113,*]

[1] *Genome Diagnostics Program, IFOM ETS - the AIRC Institute of Molecular Oncology, Milan, Italy*
[2] *Unité de Génomique Fonctionnelle, Institut de Cancérologie de l'Ouest Nantes-Angers, Angers, France*
[3] *Univ Angers, Nantes Université, Inserm, CNRS, CRCI2NA, SFR ICAT, Angers, France*
[4] *Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy*
[5] *IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy*
[6] *Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark*
[7] *Department of Pharmacy and Biotechnology (FaBit), University of Bologna, Bologna, Italy*
[8] *Unit of Predictive Medicine, Molecular Bases of Genetic Risk, Department of Experimental Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, Italy*
[9] *Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda MD, USA*
[10] *Fred A. Litwin Center for Cancer Genetics, Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital, Toronto, Ontario, Canada*
[11] *Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada*
[12] *Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK*
[13] *Department of Gynecology and Obstetrics, Comprehensive Cancer Center Erlangen-EMN, Friedrich-Alexander University Erlangen-Nuremberg, University Hospital Erlangen, Erlangen, Germany*
[14] *Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany*
[15] *Institute of Biochemistry and Genetics of the Ufa Federal Research Centre of the Russian Academy of Sciences, Ufa, Russia*
[16] *Department of Radiation Oncology, Hannover Medical School, Hannover, Germany*
[17] *IEO, European Institute of Oncology IRCCS, Division of Cancer Prevention and Genetics, Milan, Italy*
[18] *Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum, Bochum, Germany*
[19] *Department of Internal Medicine and Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA*
[20] *Centre for Genomic and Experimental Medicine, Institute of Genetics & Cancer, University of Edinburgh, Edinburgh, UK*
[21] *Oncólogy and Genétics Group, Instituto Investigación Sanitaria Galicia Sur-SERGAS, Vigo, Spain*
[22] *Intermountain Healthcare, Salt Lake City, UT, USA*
[23] *Cancer Epidemiology Group, University Cancer Center Hamburg (UCCH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany*
[24] *Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, Oslo, Norway*
[25] *Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway*
[26] *Department of Research, Vestre Viken Hospital, Drammen, Norway*
[27] *Section for Breast- and Endocrine Surgery, Department of Cancer, Division of Surgery, Cancer and Transplantation Medicine, Oslo University Hospital-Ullevål, Oslo, Norway*
[28] *Department of Radiology and Nuclear Medicine, Oslo University Hospital, Oslo, Norway*
[29] *Department of Pathology, Akershus University Hospital, Lørenskog, Norway*
[30] *Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway*
[31] *Department of Oncology, Division of Surgery, Cancer and Transplantation Medicine, Oslo University Hospital-Radiumhospitalet, Oslo, Norway*
[32] *National Advisory Unit on Late Effects after Cancer Treatment, Oslo University Hospital, Oslo, Norway*
[33] *Department of Oncology, Akershus University Hospital, Lørenskog, Norway*
[34] *Oslo Breast Cancer Research Consortium, Oslo University Hospital, Oslo, Norway*
[35] *Department of Medical Genetics, Oslo University Hospital and University of Oslo, Oslo, Norway*
[36] *Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden*
[37] *Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands*
[38] *Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands*
[39] *Department of Gynecology and Obstetrics, Comprehensive Cancer Center Lower Saxony, Hannover Medical School, Hannover, Germany*
[40] *Centre for Cancer Genetic Epidemiology, Department of Oncology, University of Cambridge, Cambridge, UK*
[41] *Division of Evolution, Infection and Genomics, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK*
[42] *Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester, UK*
[43] *Usher Institute of Population Health Sciences and Informatics, The University of Edinburgh, Edinburgh, UK*
[44] *Cancer Research UK Edinburgh Centre, The University of Edinburgh, Edinburgh, UK*
[45] *Cancer Genetics and Epidemiology Group, Genomic Medicine Group, Fundación Instituto de Investigación Sanitaria de Santiago de Compostela (FIDIS), Complejo Hospitalario Universitario de Santiago, SERGAS, Santiago de Compostela, Spain*
[46] *Human Genotyping Unit-CeGen, Spanish National Cancer Research Centre (CNIO), Madrid, Spain*
[47] *Centre for Biomedical Network Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III, Madrid, Spain*
[48] *Team "Exposome and Heredity", CESP, Gustave Roussy, INSERM, University Paris-Saclay, UVSQ, Villejuif, France*
[49] *Department of Cancer Genetics, Therapeutics and Ultrastructural Pathology, The Cyprus Institute of Neurology & Genetics, Nicosia, Cyprus*
[50] *Center for Familial Breast and Ovarian Cancer, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany*
[51] *Center for Integrated Oncology (CIO), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany*
[52] *Molecular Genetics of Breast Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany*
[53] *Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, the Netherlands*
[54] *Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany*
[55] *University of Tübingen, Tübingen, Germany*
[56] *Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, Victoria, Australia*
[57] *Research Department, Peter MacCallum Cancer Center, Melbourne, Victoria, Australia*
[58] *Department of Genetics and Pathology, International Hereditary Cancer Center, Pomeranian Medical University, Szczecin, Poland*
[59] *Independent Laboratory of Molecular Biology and Genetic Diagnostics, Pomeranian Medical University, Szczecin, Poland*
[60] *Unit of Medical Genetics, Department of Medical Oncology and Hematology, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Italy*
[61] *Department of Medical Oncology, University Hospital of Heraklion, Heraklion, Greece*
[62] *Cancer Epidemiology Division, Cancer Council Victoria, Melbourne, Victoria, Australia*
[63] *Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Victoria, Australia*
[64] *Precision Medicine, School of Clinical Sciences at Monash Health, Monash University, Clayton, Victoria, Australia*
[65] *Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[66] *Laboratory Medicine Program, University Health Network, Toronto, Ontario, Canada*
[67] *Institute for Medical Biometry and Epidemiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*
[68] *Institute for Occupational and Maritime Medicine (ZfAM), University Medical Center Hamburg-Eppendorf, Hamburg, Germany*
[69] *Department of Comparative Biomedical Sciences, College of Veterinary Medicine, Mississippi State University, Starkville, Mississippi, USA*
[70] *Department of Basic Sciences, Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH & RC), Lahore, Pakistan*
[71] *Division of Oncology, Faculty of Medicine, School of Health Sciences, University of Thessaly, Larissa, Greece*
[72] *School of Cancer & Pharmaceutical Sciences, Comprehensive Cancer Centre, Guy's Campus, King's College London, London, UK*
[73] *Center for Molecular Medicine Cologne (CMMC), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany*
[74] *Department of Clinical Pathology, The University of Melbourne, Melbourne, Australia*

[75] Public Health Program, QIMR Berghofer, Brisbane, Queensland, Australia
[76] Department of Oncology, Old Road Campus Research Building, University of Oxford, Oxford, UK
[77] Department of Oncology, Södersjukhuset, Stockholm, Sweden
[78] Department of Clinical Science and Education, Södersjukhuset, Karolinska Institutet, Stockholm, Sweden
[79] Division of Biostatistics, Data Science Institute, and Cancer Center, Medical College of Wisconsin, Milwaukee, WI, USA
[80] Department of Quantitative Health Sciences, Mayo Clinic, Rochester, USA
[81] Department of Population Science, American Cancer Society, Atlanta, USA
[82] Department of Radiology, University of Wisconsin School of Medicine and Public Health, Madison, USA
[83] Department of Population and Public Health Sciences, Keck School of Medicine, University of Southern California, USA
[84] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, USA
[85] Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA
[86] Departments of Epidemiology and Nutrition, Harvard T.H. Chan School of Public Health, Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, USA
[87] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, USA
[88] University of Washington, Seattle, USA
[89] University of California, San Diego, La Jolla California, USA
[90] Beckman Research Institute of City of Hope, Duarte, USA
[91] Epidemiology Branch, National Institute of Environmental Health Sciences, NIH, Durham NC, USA
[92] Mayo Clinic, Rochester, USA
[93] Slone Epidemiology Center, Boston University Chobanian & Avedisian School of Medicine, Boston, USA
[94] Biostatistics and Computational Biology Branch, National Institute of Environmental Health Sciences, NIH, Durham NC, USA
[95] The University of Kansas Cancer Center, Kansas City, KS USA
[96] Department of Medical Oncology, Mayo Clinic, Rochester, USA
[97] Roswell Park Cancer Center, Buffalo, USA
[98] Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[99] Institute of Pathological Physiology, First Faculty of Medicine, Charles University, Prague, Czech Republic
[100] Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[101] Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia
[102] Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, Victoria, Australia
[103] Parkville Familial Cancer Centre, Peter MacCallum Cancer Centre and Royal Melbourne Hospital, Melbourne, Australia
[104] Clinical Genomics, School of Translational Medicine, Monash University, Melbourne, Australia
[105] Inserm, U1331, Paris, France
[106] Institut Curie, Paris, France
[107] Mines Paris, Fontainebleau, France
[108] PSL Research University, Paris, France
[109] Institut Curie, Service de Génétique, Paris, France
[110] Inserm, U1339, Paris, France
[111] Université Paris Cité, Paris, France
[112] Molecular Oncology Laboratory, Hospital Clinico San Carlos, Instituto de Investigación Sanitaria San Carlos (IdISSC), Madrid, Spain
[113] Laboratory of Hematology-Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy

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ABSTRACT

The breast cancer risk conferred by germline protein truncating variants (PTVs) in known and putative breast cancer genes has been extensively investigated. However, the effect of *FANCM* PTVs on breast cancer risk remains unclear. Our previous clinical, genetic and functional results on the N-terminal p.Arg658* and the two C-terminal p.Gln1701* and p.Gly1906Alafs*12 variants suggested that *FANCM* PTVs may confer different risks for ER-negative (ER-neg) and triple-negative (TN) breast cancer subtypes.

Here, we performed meta-analyses of seven studies totaling 144 681 breast cancer cases and 123 632 controls. *FANCM* PTVs were tested for association with breast cancer risk overall and the disease clinical subtypes by single variant and burden analyses. Two CRISPR-Cas9-based functional assays were also conducted to test the fitness of cells after knock-in of the p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12 PTVs and the sensitivity of different FANCM regions to genome editing.

Our results suggest that the N-terminal FANCM region upstream of p.Tyr725 harbors essential functions, whereas downstream regions appear dispensable. This is supported by our genetic data which indicate that all *FANCM* PTVs, excluding the two C-terminal p.Gln1701* and p.Gly1906Alafs*12, are associated with an increased risk of ER-neg (OR = 1.41, $P = 0.023$) and TN (OR = 1.64, $P = 0.0023$). Notably, PTVs upstream of AA position 670 are associated with a moderate risk of developing TN breast cancer, and that even when the p.Arg658* carriers were excluded from the analysis. Importantly, our results confirm previous data indicating that p.Arg658* carriers are at moderate risk of developing ER-neg (OR = 2.08, P = 0.030) and TN (OR = 3.26; P = 0.0034), whereas carriers of p.Gln1701* and p.Gly1906Alafs*12 should not be considered at increased risk. Our data are useful for counseling carriers of *FANCM* PTVs, but further analyses are warranted to obtain more precise risk estimates.

1. Introduction

Many genes have been proposed as risk factors for breast cancer, but few have been established with convincing evidence. Protein truncating variants (PTVs) and some missense variants in *BRCA1, BRCA2,* and *PALB2* are known to be high-penetrance risk factors for breast cancer, and there is a growing consensus that i) PTVs and some missense variants in *BARD1*, *RAD51C,* and *RAD51D* are moderate risk factors for estrogen receptor-negative (ER-neg) breast cancer; ii) PTVs in *ATM* and *CHEK2* are moderate risk factors for ER-positive (ER-pos) breast cancer; and iii) both missenses and PTVs in *TP53* are associated with breast

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[#] Co-first authors

cancer risk, although the magnitude of the risk remains to be determined [1–4]. Other breast cancer genes have recently emerged and require further genetic and functional validation.

The *FANCM* gene encodes a 2048 amino acid (AA) sized protein involved in DNA replication stress response and inter-strand cross-link repair mediated by the Fanconi anemia (FA) pathway [5,6]. *FANCM* was first implicated in breast cancer risk when the c.5791C > T PTV, known as p.Arg1931* but re-annotated as p.Gly1906Alafs*12 due to its spliceogenic effect, was detected in a breast cancer family [7,8]. Subsequently, case-control studies were performed — including one in non-Finnish Caucasians familial cases with no *BRCA1* and *BRCA2* pathogenic variants [9] — but the association of *FANCM* PTVs with overall breast cancer risk was not consistently found [10]. Recently, two very large studies were conducted. BRIDGES reported a borderline association between *FANCM* PTVs and the risk of ER-neg and triple-negative (TN) breast cancer subtypes [1], whereas CARRIERS found no significant association across different subgroups possibly due to its reduced statistical power [2]. We have previously evaluated the three most common PTVs p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12. We initially observed that the C-terminal p.Gly1906Alafs*12 was associated with risk for familial breast cancer (OR = 3.67, *P* = 0.043) [11]. Later, analyzing studies that were predominantly population-based, we found that the N-terminal p.Arg658* was associated with a moderate risk of ER-neg (OR = 2.44, *P* = 0.034) and TN (OR = 3.79, *P* = 0.009), whereas no association was found for p.Gln1701* and an inconsistent association was found between p.Gly1906Alafs*12 and ER-neg breast cancer [11,12]. In addition, clinical observations suggested that p.Arg658* may confer a higher breast cancer risk and more severe cellular phenotypes than the C-terminal p.Gln1701* and p.Gly1906Alafs*12 [13,14].

In the present study, we aimed to clarify the effect of *FANCM* PTVs on breast cancer risk by conducting single variant and burden analyses. To this end, we performed meta-analyses of seven case-control studies including clinical and gene variant sequencing data from a total of 268 313 women. Additionally, we performed CRISPR-Select assays to evaluate the impact of p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12 on cell survival, and we agnostically investigated the essentiality of different *FANCM* regions using a CRISPR-Cas9-based assay.

2. Materials and methods

2.1. Meta-analyses of genetic data from case-control studies

In these analyses, we included data from seven studies [1,2,11,15–18] totaling 144 681 breast cancer cases and 123 632 controls (Table 1). For some of these studies, analysis results of the association between *FANCM* PTVs and breast cancer risk were previously published. Raw data were obtained from the BRIDGES, OncoArray and GENESIS studies, whereas summary statistics from locally performed analyses were available for the remaining four studies. For each study, odds ratio (OR) with 95 % confidence intervals (CIs) and *P-value* (*P*) for PTV carriers compared with non-carriers were estimated using univariable logistic regression. PTVs were analyzed either individually (p.Arg658*, p.Gln1701*, and p.Gly1906Alafs*12) or combined in burden analyses. In these statistical analyses, breast cancer cases were tested combined or based on their classification, when known, into ER-neg, ER-pos and TN breast cancer subgroups.

Meta-analyses were performed by combining the ORs from each study. We applied fixed-effect models when no substantial heterogeneity was observed across studies ($I^2<30$ %), whereas random-effects models with restricted maximum likelihood (REML) estimation were used when heterogeneity was higher ($I^2\geq 30$ %). Statistical analyses were performed using STATA version 15.1 (StataCorp LLC, College Station, Texas, USA) and the R software. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

2.2. CRISPR-Select^{TIME} assay

The CRISPR-Select^{TIME} assay was used as previously described [19]. For one 9.6 cm^2 well, 60 pmol each of crRNA (Supp.Table 1) and tracrRNA were mixed and allowed to complex for 10 min. One hundred and twenty-five μl of OptiMEM were added to the mixture followed by 10 pmol of each of the variants under analysis and of the synonymous internal normalization variant (WT') single-stranded oligo DNAs (ssODNs) and Lipofectamine (Thermo Fisher Scientific) before dripping on iCas9-MCF10A cells (TP53 WT). On days 2 and 12 post-transfection, cell population aliquots were collected for the variant:WT' ratio analysis. Genomic DNA was extracted from cell population aliquots using GenElute Mammalian Genomic DNA Miniprep (Sigma). Two rounds of PCR amplifications (Supp.Table 1) were performed to prepare the products for NGS. Finally, an amplicon sequencing library was generated using MiSeq Reagent kit V2 (Illumina) and sequenced on a MiSeq instrument (Illumina). The NGS data were analyzed using the CRISPResso2 online tool (http://crispresso.pinellolab.org). The frequencies of frameshift indels represented knockout efficiency and the frequencies of each edited variant represented knock-in efficiency.

2.3. Sensitivity of FANCM to genome editing

We used the haploid HAP1^{LIG4-} cell line as previously described

Table 1
Description of the breast cancer case-control studies included in this analysis.

Study - reference (FANCM PTVs tested)	Controls	All cases	ER-positve cases	ER-negative cases	TNBC cases	Population	Study type
OncoArray - Figlioli G et al., 2019 (p.Arg658*, p.Gln1701*, p.Gly1906Alafs*12)	53 766	67 112	44 565	10 770	4805	Europeans	Population- and family-based
GENESIS - Girard E et al., 2019 (all PTVs)	1199	1207	659	129	36	French (95 % European)	Family-based
BRIDGES - BCAC et al., 2021 (all PTVs[a])	37 645	42 013	23 596	6182	2309	Europeans	Population- and family-based
excluding overlaps with OncoArray	18 326	25 297	13 279	3375	1219		
CARRIERS - Hu C et al., 2021 (all PTVs[a])	32 465	32 326	20 949	4281	1446	US (75 % European)	Population-based
BEACCON - Li N et al., 2021 (all PTVs)	14 577	6809	2169	1168	791	Australians (95 % European)	Family-based
ABCFS - Southey M et al., 2021 (all PTVs)	846	1359	964	395	not tested	Australians	Population-based
CZECANCA - Soukupova et al., 2018 (all PTVs)	2453	10 571	5321	2218	1452	Czechs (100 % European)	Family-based
All Studies	123 632	144 681	87 906	22 336	9749	Prevalently Europeans	Population- and family-based

[a] Truncating variants at the end of the penultimate exon or the last exon that potentially avoid nonsense mediated mRNA decay and do not influence known functional domains were excluded.

(LIG4 KO clone#5 in Ref. [20]) in which *FANCM* is essential [21]. Therefore, if essential functions of the gene are disrupted, these cells are expected to show a loss of fitness or die. Twelve crRNA (Integrated DNA Technologies) were designed to target six *FANCM* regions (Supp. Table 1). Each of the crRNAs was annealed with the common tracrRNA (Integrated DNA Technologies) at a final concentration of 1 μM and complexed with 6 pmol of Cas9 Nuclease (Integrated DNA Technologies) in OptiMEM. Each crRNA was named according to the predicted AA position of the Cas9-mediated double-stranded break (DSB). Pre-assembled ribonucleoprotein complexes were transfected into HAP1^{LIG4-} cells using Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Thermo Fisher Scientific). Cells were cultured for 7 days before limited dilution. Approximately 30 clones were amplified for each of the fourteen transfections. DNA was extracted from each clone using Gitschier buffer and 0.5 mg/mL Proteinase K and subjected to PCR amplification (Supp.Table 1). Amplified DNAs were subjected to Sanger sequencing and analyzed using Geneious, TIDE software [22] and InDelphi algorithm [23]. Each of the 12 transfections was repeated three times and a total of 1045 clones were annotated.

3. Results

3.1. Meta-analyses of case-control studies

We conducted meta-analyses combining data from seven case-control studies (Table 1). First, we assessed the effect of the three most common PTVs — p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12 — on breast cancer risk. Carriers of the p.Arg658* PTV showed a significantly increased risk of ER-neg (OR = 2.08, 95 % CI 1.07–4.02, *P* = 0.030) and TN (OR = 3.26, 95 % CI 1.48–7.20, *P* = 0.0034) breast cancer subtypes. Triple-negative breast cancer cases are included in the ER-neg group; hence, it should be noted that the risk effect observed in the ER-neg group could be seen as a diluted effect of the risk observed in the TN breast cancer group. In contrast, the C-terminal p.Gln1701* and p.Gly1906Alafs*12 PTVs showed no association with overall breast cancer risk or with the risks of ER-neg, ER-pos and TN (Fig. 1 and Supp. Table 2).

Then, we performed a burden analysis to assess the effect of any *FANCM* PTV on overall breast cancer risk, and on ER-pos, ER-neg, and TN subtypes. Carriers of *FANCM* PTVs had an increased risk of developing ER-neg breast cancer (OR = 1.38, 95 % CI 1.10–1.72, *P* = 0.0047). However, no association was observed between all PTVs and overall breast cancer risk, or the ER-pos and TN subtypes (Fig. 2 and Sup. Table 2).

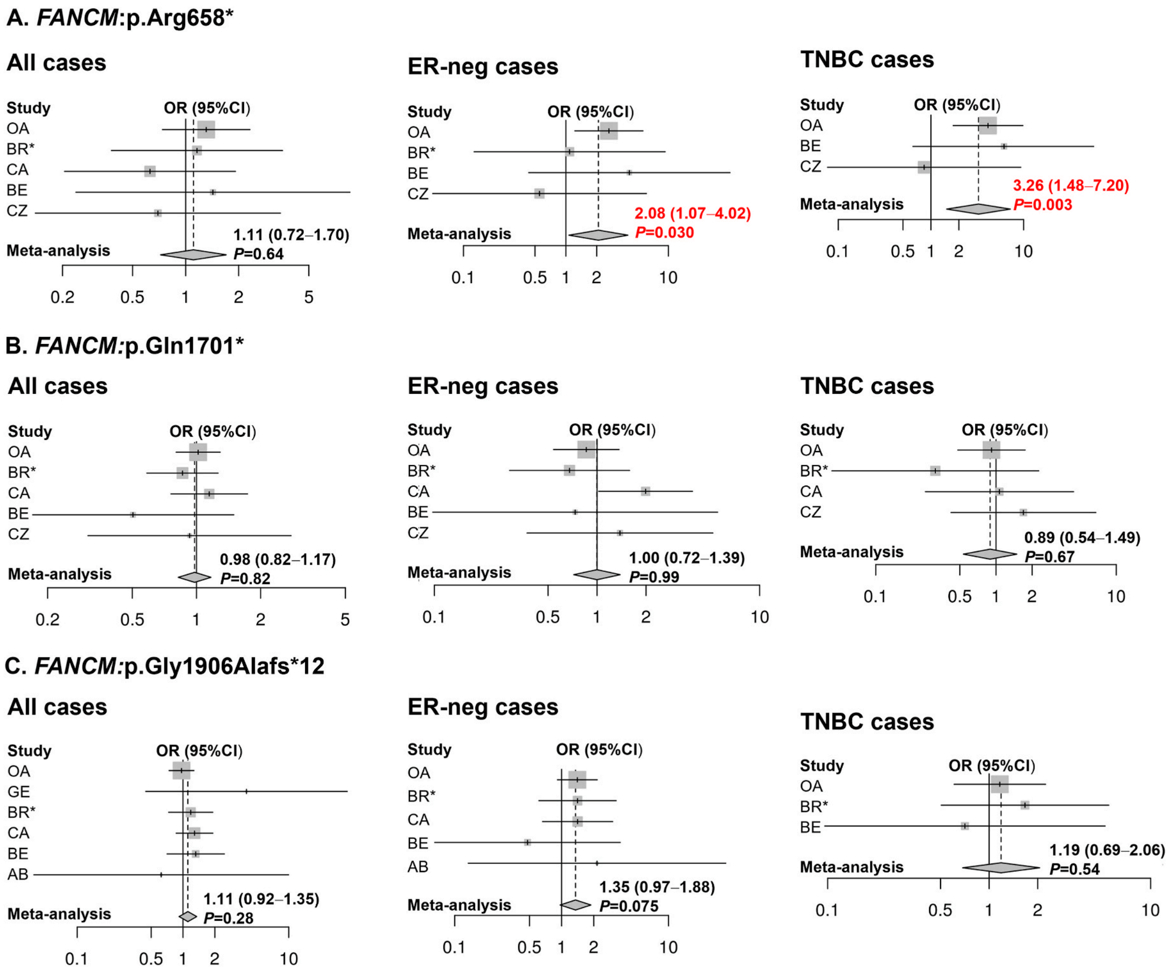

Fig. 1. Meta-analysis of studies testing the association of (**A**) *FANCM*:p.Arg658*, (**B**) p.Gly1906Alafs*12, and (**C**) p.Gln1701* with overall breast cancer risk and in the ER-negative and triple negative (TN) disease subtypes. For each study (AB, ABCFS; BE, BEACCON; BR, BRIDGES; CA, CARRIERS; CZ, CZECANCA; GE, GENESIS; OA, OncoArray), the odds ratio (OR) and 95 % confidence intervals (CIs) are shown by a vertical dash and horizontal line, respectively. The grey boxes indicate the weight of each study. Meta-analyses results are shown by grey diamonds. Statistically significant results are reported in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

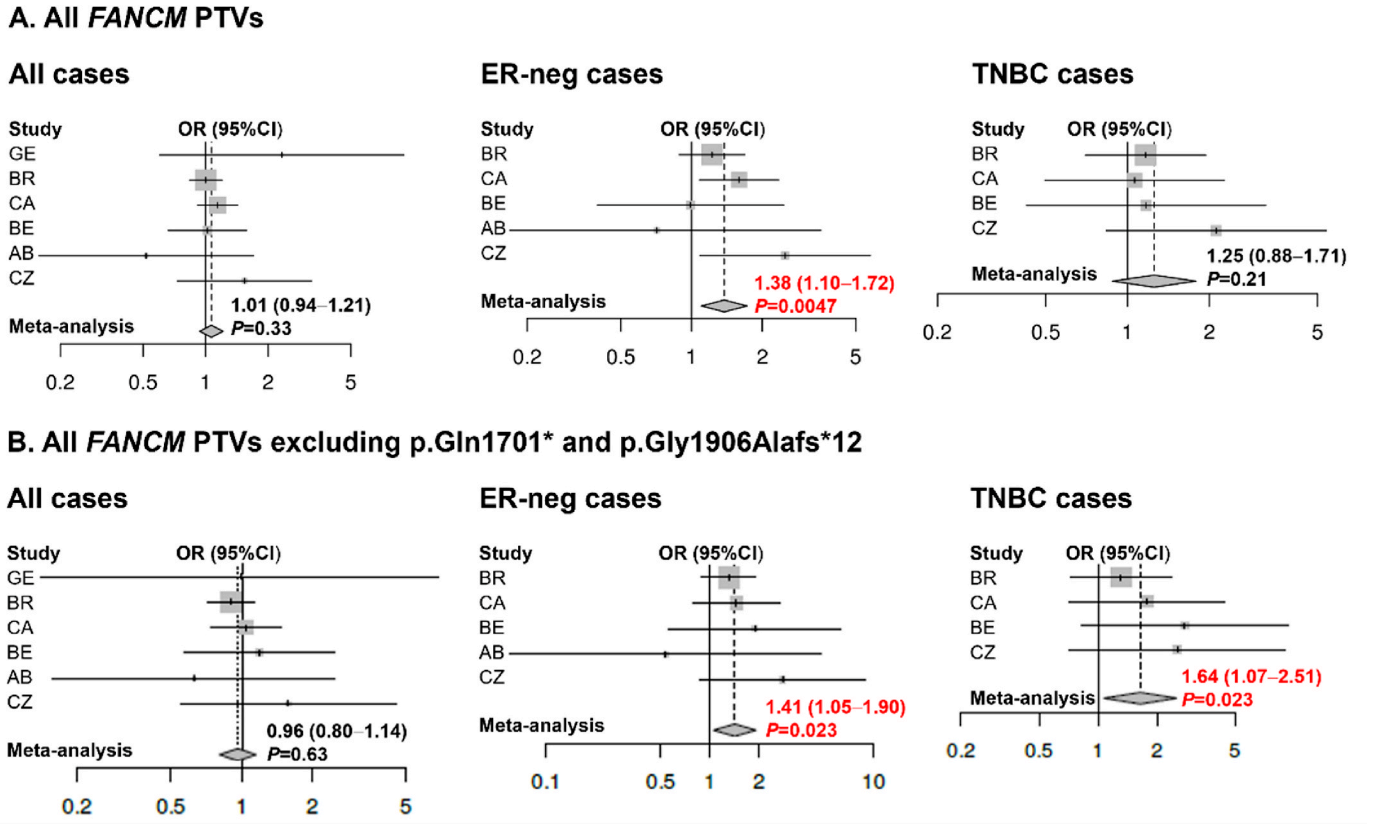

Fig. 2. Meta-analysis of studies testing the association of (**A**) all *FANCM* PTVs and (**B**) all *FANCM* PTVs excluding p.Gln1701* and p.Gly1906Alafs*12 with overall breast cancer risk and in the ER-negative and triple negative (TN) disease subtypes. For each study (AB, ABCFS; BE, BEACCON; BR, BRIDGES; CA, CARRIERS; CZ, CZECANCA; GE, GENESIS), the odds ratio (OR) and 95 % confidence intervals (CIs) are shown by a vertical dash and horizontal line, respectively. The grey boxes indicate the weight of each study. Meta-analyses results are shown by black grey diamonds. Statistically significant results are reported in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

We repeated the burden analysis excluding p.Gln1701* and p.Gly190Alafs*12. In this analysis, carriers of any of the remaining *FANCM* PTVs showed an increased risk of developing ER-neg ($OR = 1.41$, 95 % CI 1.05−1.90, $P = 0.023$) and TN ($OR = 1.64$, 95 % CI 1.07−2.51, $P = 0.0023$, Fig. 2 and Supp.Table 2), but not ER-pos or breast cancer overall. Finally, we performed an additional burden analysis excluding p.Arg658* and found that carriers of any of the remaining *FANCM* PTVs are at increased risk of developing ER-neg breast cancer ($OR = 1.43$, 95 % CI 1.05−1.93, $P = 0.022$, Supp.Table 2).

3.2. CRISPR-Select^{TIME} functional assay on the three most common PTVs

We performed the CRISPR-Select^{TIME} assay to investigate the effect of the p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12 PTVs on iCas9-MCF10A cells (Fig. 3). This quantitative assay tracks the frequency of the tested variants over time relative to a neutral variant (WT') that serves as an internal control [19]. First, we tested the common variant p.Asn655Ser (c.1964A > G) [24] as a negative control and the p.Arg200* (c.597A > G) PTV as a positive control. As expected, there was no significant change in the variant/WT' ratio for p.Asn655Ser and a decrease in the variant/WT' ratio for p.Arg200* (Fig. 3 and Supp.Figure 1). When we tested the p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12 PTVs, we observed a decrease in the variant/WT' ratio for all of the PTVs, but with a statistically significant reduction for p.Arg658* and p.Gly1906Alafs*12 (48 % and 33 % respectively) (Fig. 3). We obtained knock-in efficiencies of 1.84 % and 3.13 % for p.Arg658* and Gly1906Alafs*12, respectively (Supp.Figure 1). The lower knock-in efficiency of 0.56 % for p.Gln1701* was compensated by the reproducibility of the replicate results. Taken together, these data suggest that p.Arg658* is functionally more deleterious than the p.Gln1701* and possibly of p.Gly1906Alafs*12.

3.3. FANCM sensitivity assay in HAP1^{LIG4-} cells

We then tested the gene agnostically by knocking out different regions, using haploid HAP1^{LIG4-} cells in which *FANCM* is essential (Fig. 4A) [21]. In this assay, for each of the 12 crRNAs used (Fig. 4B), we compared the observed "clone genotype patterns" with the DNA repair outcomes of Cas9-induced DSBs predicted by the computational model "InDelphi" [23,25] (Fig. 4C and Supp.Figure 2). We noted that all the observed recurrent in-frame indels were among the most frequently predicted by InDelphi and that frameshift events were predicted at all the 12 targeted positions.

We observed that in the three N-terminal regions AA 171–199, AA 516–522, and AA 631–658, only one KO clone was detected out of 515 annotated clones. In contrast, in the three C-terminal regions AA 725–746, AA 1257–1260 and AA 1675–1701, 83 KO clones were detected out of 530 annotated clones ($P < 0.0001$, Fig. 4C and Supp. Table 3). Furthermore, we noted that short in-frame indels (<21bp) were present in all targeted regions with 123 annotated clones upstream and 124 downstream of AA position 659, respectively. On the contrary, as for the KO clone distribution, long (>54bp) in-frame indels were predominantly observed downstream of AA position 658 with 37 annotated clones and only one upstream of this limit ($P < 0.0001$, Fig. 4C and Supp.Table 3). These findings suggest that *FANCM* N-terminal region upstream of the AA position 659 is essential, whereas the C-terminal region downstream of AA position 724 is not.

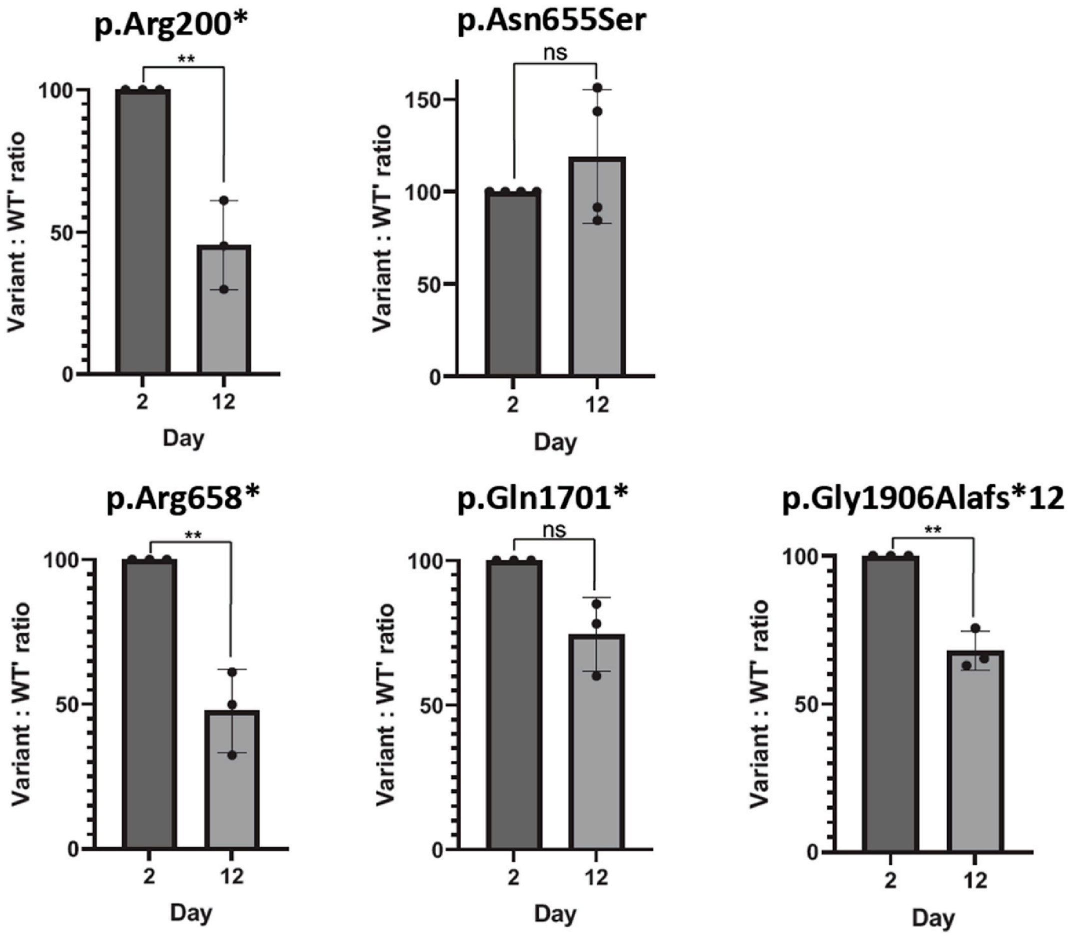

Fig. 3. CRISPR-Select$^{\text{TIME}}$ functional assay. The Variant:WT' ratios were determined at the indicated day 2 and 12 time points. The Day 12 time points are normalised to the respective Day 2 time points. Data are means ± s.d. of n = 3 or n = 4 independent biological replicates and have been analyzed by two-tailed paired *t*-test. **(P $<$ 0.005), ns = not significant ($P > 0.05$).

3.4. *Meta-analyses of case-control studies assessing the effect of N-terminal and C-terminal FANCM PTVs*

Based on the results of our functional analyses, we wanted to test whether or not N-terminal and C-terminal PTVs have different effects on breast cancer risk. We identified a boundary between essential and non-essential regions of *FANCM* located between AA position 658 and 725, which could be compatible with the existence of a shorter *FANCM* mRNA isoform (NM_001308134.2; ENST00000556036.5) encoding a 669 AA long protein. Therefore, N-terminal and C-terminal PTVs were sub-grouped according to this cut-off.

We observed a significant association between only N-terminal PTVs and the risk of TN breast cancer (PTVs located upstream the AA position 670: OR = 2.05, 95 % CI 1.04–4.03; *P* = 0.0037), whereas no significant associations were found for C-terminal PTVs (PTVs located downstream the AA position 671, Supp.Table 2). Furthermore, in the analysis of upstream PTVs excluding p.Arg658*, we confirmed the significant association with TN breast cancer risk (OR = 2.87, 95 % CI 1.36–6.06; *P* = 0.006), further supporting the importance of the N-terminal *FANCM* region.

4. Discussion

In this study, we conducted meta-analyses of case-control studies to clarify the association between *FANCM* PTVs and breast cancer risk. Our findings indicate that, among the common *FANCM* PTVs, p.Arg658*, p.Gln1701* and p.Gly1906Alafs*12, only p.Arg658* resulted associated with a moderate risk of developing ER-neg and TN breast cancer subtypes (Fig. 1). It should be noted that we previously tested these three PTVs in the OncoArray study [11]. In the present single-PTV meta-analyses, additional studies were combined with OncoArray; however, their combined weight was generally limited varying between 27.7 and 47.3 % (Supp. Table 2).

In the CRISPR-Select$^{\text{TIME}}$ assay, we consistently observed that the effect of the p.Arg658* on loss of cellular fitness was significantly stronger than that of p.Gln1701* and p.Gly1906Alafs*12 (Fig. 3). Overall, these results corroborate our previously published data showing that p.Arg658* is a moderate risk factor for ER-neg and TN and is associated with reduced cell survival and increased number of cell chromatid breaks compared to p.Gln1701* and p.Gly1906Alafs*12 [11]. As further evidence of this reduced effect of p.Gln1701* and p.Gly1906Alafs*12 on breast cancer risk, more convincing associations for both ER-neg and TN were observed when these two variants were excluded from the analysis of all PTVs combined (Fig. 2). As these two variants also account for approximately 50 % of all *FANCM* PTVs, their inclusion is likely to have diluted the overall association between *FANCM* PTVs and breast cancer risk, which may explain the BRIDGES and CARRIERS results.

Furthermore, the sensitivity to genome editing indicated that two different FANCM regions exist (Fig. 4C). The first region, from AA 171 to 658, contains the entire DEAH domain and does not tolerate KO, suggesting that it harbors essential functions. The second region, from AA position 725, tolerates KO suggesting that it contains non-essential functions. This may be explained by the existence of a shorter *FANCM*

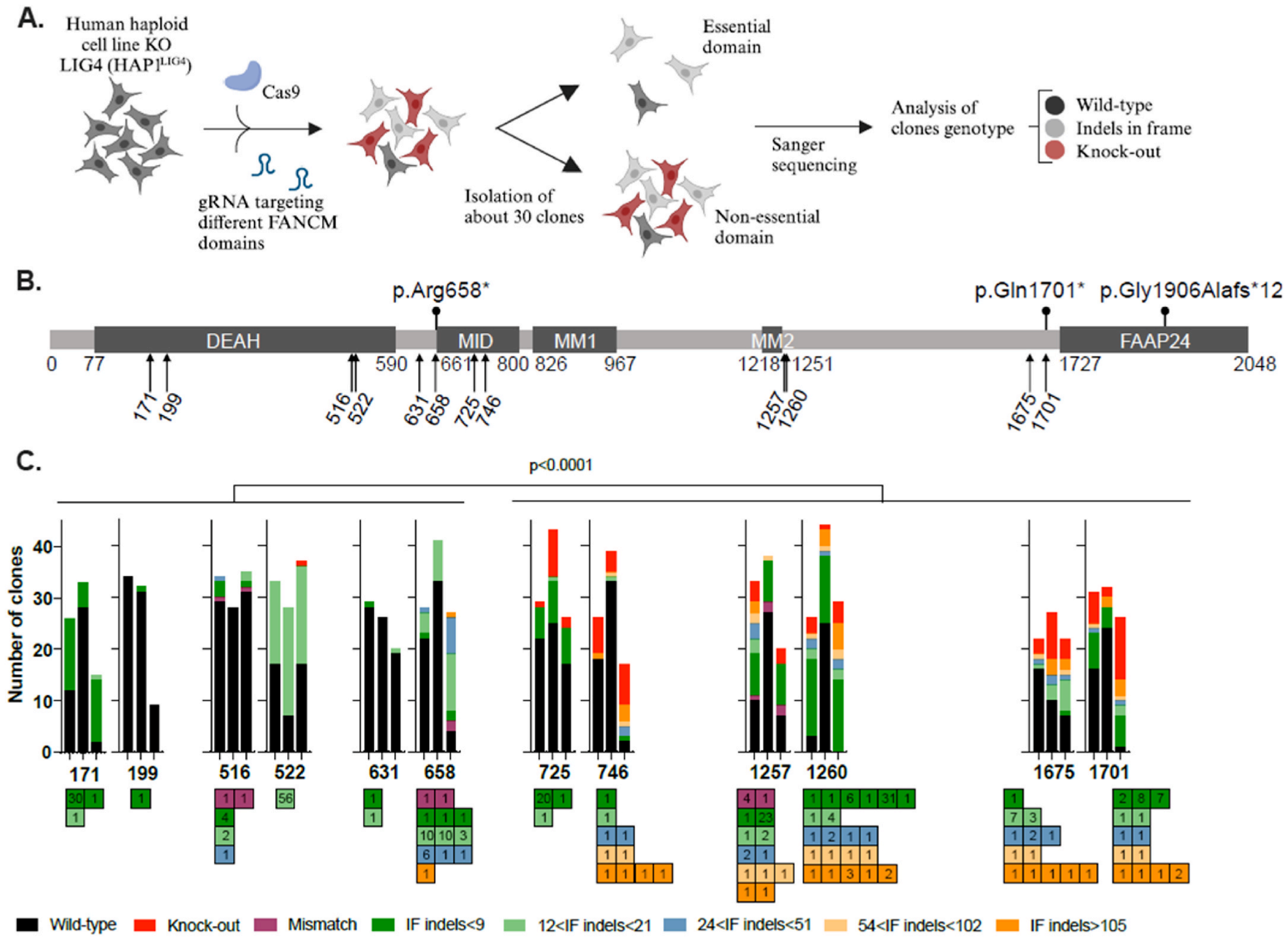

Fig. 4. Study of the essentiality of the FANCM regions in HAP1^{LIG4-} cells. (**A**) Schematic description of the CRISPR/Cas9-based assay used (created with BioRender). (**B**) Schematic representation of the 2048 AA FANCM protein and its conserved domains: DEAH, ATPase-dependent DNA translocase domain; MID, MHF1/MHF2 interaction domain; MM1, FA core complex interaction domain; MM2, Bloom's complex interaction domain; PND, interacting with FAAP24. The positions of the three most common PTVs are indicated by dots. The positions of the 12 crRNAs used to target six *FANCM* regions are indicated by arrows. (**C**) Representation of the 1045 clones obtained for each of the 12 targeted AA positions, with the three replicates shown according to their genotypes. Each square represents the number of clones with an identical in-frame indel length or the number of clones with mismatch variants. The difference in the proportions of KO clones detected in the targeted regions at the gene 5′-end (spanning AA 161 to 658) compared to those detected in more distal gene regions (spanning AA 725 to 1701) was tested and found to be significatively different using two-way ANOVA tests, ($p < 0.0001$).

mRNA isoform (NM_001308134.2; ENST00000556036.5) encoding a 669 AA sized protein resulting from alternative RNA processing that causes the retention of a portion of the intron 11 containing a stop codon. Notably, short FANCM fragments containing only the N-terminal translocase domain have been shown to retain affinity for branched DNA, as well as ATPase activity and R- and D-loop resolution [26–29]. It is known that mRNA transcripts carrying a premature termination codon (PTC) are expected to be completely or partially degraded by nonsense-mediated decay (NMD) unless the PTCs are located in the last exon or in the 3′-most 50 bp of the penultimate exon [30]. Accordingly, we argue that *FANCM* PTVs may induce different NMD effects on the full-length and short mRNA isoforms, depending on their location. In particular, we hypothesize that p.Arg658* would affect both the short and full-length isoforms. The p.Gln1701* would not affect the expression of the short isoform but would cause the full-length isoform to be degraded, at least partially, by NMD. The p.Gly1906Alafs*12 alters normal splicing by introducing a PTC which, due to its position, is expected to escape NMD [8]. This PTV would therefore result in the expression of a normal short isoform and of a protein truncated in the FAAP24 interacting domain. This might explain the different levels of breast cancer risk observed. Moreover, the N-terminal domain of FANCM was recently found to have a dual role [31]. In this context, the essentiality of ATP-dependent branch migration activity for DNA damage survival, but not its involvement with the FA core complex, could be link to our observation since we observed that only the DEAH domain of FANCM was essential but not the entire MM1 domain.

To further investigate the differential essentiality of FANCM functions, we assessed the effect of PTVs located upstream or downstream of AA position 670 on breast cancer risk (Supp.Table 2). We only identified a significant association between upstream PTVs and the risk of TN breast cancer. Notably, the analysis of upstream PTVs excluding p.Arg658* revealed a particularly strong association with TN breast cancer (OR = 2.87), suggesting that even relatively rarer N-terminal truncations may confer substantial risk for this aggressive breast cancer subtype. The lack of association with the risk of ER-neg breast cancer was however unexpected, as p.Arg658* alone was associated with an increased risk of both ER-neg and TN breast cancer (Fig. 1). However, the analysis of PTVs upstream of AA position 670 was based on a smaller sample size compared to the analysis of the p.Arg658*, which may explain this discrepancy. Therefore, the analysis of PTVs upstream of AA position 670 may not have sufficient statistical power to detect the true effect of these PTVs on ER-neg breast cancer risk. Accordingly, it is noteworthy that the ORs found for PTVs upstream of AA 670 in overall breast cancer, ER-neg and TN cases showed an increasing trend, suggesting a potential true association that requires further validation.

In this study, we found that *FANCM* N-terminal PTVs are associated with risk of developing ER-neg and TN breast cancer subtypes. While pathogenic variants in *BRCA1* predominantly predispose to the same subtypes, germline defect in *BRCA2* more often lead to ER-pos tumors [1,2]. All these three genes play key function in the FA/BRCA molecular pathway that is responsible for DNA repair by homologous recombination (HR). In the pathway, *FANCM* and *BRCA1* are early players that sense replication stress and coordinate DNA HR repair initiation, whereas *BRCA2* functions later, in *RAD51* filament stabilization [31,32]. Hence, it could be speculated that this mechanistic difference might explain the effects of *FANCM*, *BRCA1* and *BRCA2* on the risks of developing specific breast cancer subtypes. Additionally, cells deficient in *BRCA1*, *BRCA2* or some other genes involved in the FA/BRCA pathway are sensitive to the poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi), which are now routinely used in personalized cancer therapies. Consistently, we and other have shown that FANCM-depleted cell lines are also hypersensitive to PARPi [11,33,34]. Therefore, it is expected that cancer patients with pathogenic variants in *FANCM* may also benefit from PARPi-based treatments.

Our study has several strengths. First, it represents the largest meta-analysis to date assessing the association between *FANCM* PTVs and breast cancer risk, pooling data from more than 270 000 women across multiple large-scale cohort studies, most of which had previously published high-quality data. This unprecedented sample size substantially increased statistical power and enabled more precise risk estimates across breast cancer subtypes. Second, we complemented the genetic evidence with two different robust functional assays based on CRISPR/Cas9 technologies. These experiments highlighted the differential roles of N-terminal and C-terminal regions, consistently with the genetic associations results. Third, by grouping PTVs according to their genomic position, we proposed a biologically plausible mechanistic model linking FANCM functional domains with differential risks for ER-neg and TN breast cancer. Finally, the integration of multiple analytical approaches (single PTVs, burden, and positional analyses) strengthens the overall conclusions and provides a comprehensive view of FANCM's role in breast cancer predisposition. Some limitations should also be acknowledged. Because *FANCM* PTVs are rare, the number of carriers in specific subgroups was small, which reduced the statistical power of some subgroup analyses. The positional boundary, which is located between AA 670 and AA 725, was defined using functional data and is subject to refinement. For some studies, only summary-level genetic data were available rather than individual-level data, preventing more detailed analyses such as stratification by age groups or for additional covariates. Similarly, certain clinico-pathological tumor characteristics, such as HER2 status, were not consistently available across all contributing studies, limiting our ability to perform additional stratified analyses by tumor characteristics. The lack of coherent clinical information across contributing studies introduces the risk of misclassification of breast cancer subtypes, particularly for ER status, when clinical data were incomplete, inconsistently reported, or assessed with different laboratory methods. Some studies may also have used different inclusion criteria (for example, oversampling patients with strong family history of breast cancer), which could lead to overestimation of risk associations. Furthermore, the majority of the studies included women of European ancestry, which restricts the generalizability of our conclusions to other populations. Finally, this was a meta-analysis of case–control studies, and while this design is particularly suitable for studying rare exposures such as *FANCM* PTVs, residual confounding cannot be fully excluded.

5. Conclusion

Our functional results suggest that the N-terminal FANCM region upstream of AA 725 harbors essential functions, whereas downstream regions appear dispensable. Genetic data indicate that all *FANCM* PTVs, excluding the two C-terminal p.Gln1701* and p.Gly1906Alafs*12, are associated with an increased risk of developing ER-neg and TN breast cancer subtypes. Specifically, PTVs upstream of AA position 670 are associated with a moderate risk of developing TN breast cancer. Importantly, our genetic and functional results support the knowledge that carriers of p.Arg658* have a moderate risk of developing ER-neg and TN subtypes, whereas carriers of p.Gln1701* and p.Gly1906Alafs*12 appear not to be at risk for breast cancer or any disease subtype. While our data are informative for more efficient counseling of carriers of *FANCM* individuals PTVs, additional more extensive studies are warranted to better define the risk magnitude of *FANCM* PTVs.

CRediT authorship contribution statement

Amandine Billaud: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gisella Figlioli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Clémence Mooser:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Irene Casamassima:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Violette Azzoni:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Jahnavi Srivatsa:** Writing – review & editing, Investigation, Formal analysis. **Mara Colombo:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Laura Caleca:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Thomas U. Ahearn:** Writing – review & editing, Data curation. **Irene L. Andrulis:** Writing – review & editing, Data curation. **Antonis C. Antoniou:** Writing – review & editing, Data curation. **Matthias W. Beckmann:** Writing – review & editing, Data curation. **Sabine Behrens:** Writing – review & editing, Data curation. **Marina Bermisheva:** Writing – review & editing, Data curation. **Natalia V. Bogdanova:** Writing – review & editing, Data curation. **Manjeet K. Bolla:** Writing – review & editing, Data curation. **Bernardo Bonanni:** Writing – review & editing, Data curation. **Thomas Brüning:** Writing – review & editing, Data curation. **Nicola J. Camp:** Writing – review & editing, Data curation. **Archie Campbell:** Writing – review & editing, Data curation. **Jose E. Castelao:** Writing – review & editing, Data curation. **Melissa H. Cessna:** Writing – review & editing, Data curation. **Jenny Chang-Claude:** Writing – review & editing, Data curation. **Kamila Czene:** Writing – review & editing, Data curation. **Joe Dennis:** Writing – review & editing, Data curation. **Peter Devilee:** Writing – review & editing, Data curation. **Thilo Dörk:** Writing – review & editing, Data curation. **Alison M. Dunning:** Writing – review & editing, Data curation. **Mikael Eriksson:** Writing – review & editing, Data curation. **D. Gareth Evans:** Writing – review & editing, Data curation. **Peter A. Fasching:** Data curation, Writing – review & editing. **Jonine D. Figueroa:** Writing – review & editing, Data curation. **Marike Gabrielson:** Writing – review & editing, Data curation. **Manuela Gago-Dominguez:** Writing – review & editing, Data curation. **Anna González-Neira:** Writing – review & editing, Data curation. **Pascal Guénel:** Writing – review & editing, Data curation. **Andreas Hadjisavvas:** Writing – review & editing, Data curation. **Eric Hahnen:** Writing – review & editing, Data curation. **Ute Hamann:** Writing – review & editing, Data curation. **Peter Hillemanns:** Writing – review & editing, Data curation. **Antoinette Hollestelle:** Writing – review & editing, Data curation. **Maartje J. Hooning:** Writing – review & editing, Data curation. **Reiner Hoppe:** Writing – review & editing, Data curation. **Anthony Howell:** Writing – review & editing, Data curation. **Anna Jakubowska:** Writing – review & editing, Data curation. **Vessela N. Kristensen:** Writing – review & editing, Data curation. **Jan Lubiński:** Writing – review & editing, Data curation. **Michael Lush:** Writing – review & editing, Data curation. **Siranoush Manoukian:** Writing – review & editing, Data curation. **Dimitrios Mavroudis:** Writing – review & editing, Data curation. **Roger L. Milne:** Writing – review & editing, Data curation. **Anna Marie Mulligan:** Writing – review & editing, Data curation. **William G. Newman:** Writing – review & editing, Data curation. **Nadia Obi:** Writing – review & editing, Data curation. **Mihalis I. Panayiotidis:** Writing – review & editing, Data curation. **Guillermo Pita:** Writing – review & editing, Data curation. **Muhammad U. Rashid:** Writing – review & editing, Data curation. **Valerie Rhenius:** Writing – review & editing, Data curation. **Emmanouil Saloustros:** Writing – review & editing, Data curation. **Elinor J. Sawyer:** Writing – review & editing, Data curation. **Rita K. Schmutzler:** Writing – review & editing, Data curation. **Mitul Shah:** Writing – review & editing, Data curation. **Melissa C. Southey:** Writing – review & editing, Data curation. **Amanda B. Spurdle:** Writing – review & editing, Data curation. **Thérèse Truong:** Writing – review & editing, Data curation. **Qin Wang:** Writing – review & editing, Data curation. **Camilla Wendt:** Writing – review & editing, Data curation. **Paul L. Auer:** Writing – review & editing, Data curation. **Nicholas J. Boddicker:** Writing – review & editing, Data curation. **Clara Bodelon:** Writing – review & editing, Data curation. **Elizabeth S. Burnside:** Writing – review & editing, Data curation. **Fei Chen:** Writing – review & editing, Data curation. **Fergus J. Couch:** Writing – review & editing, Data curation. **Susan M. Domchek:** Writing – review & editing, Data curation. **Heather A. Eliassen:** Writing – review & editing, Data curation. **Christopher Haiman:** Writing – review & editing, Data curation. **James M. Hodge:** Writing – review & editing, Data curation. **Chunling Hu:** Writing – review & editing, Data curation. **Hongyan Huang:** Writing – review & editing, Data curation. **Sara Lindstrom:** Writing – review & editing, Data curation. **Maria Elena Martinez:** Writing – review & editing, Data curation. **Katherine L. Nathanson:** Writing – review & editing, Data curation. **Susan L. Neuhausen:** Writing – review & editing, Data curation. **Katie M. O'Brien:** Writing – review & editing, Data curation. **Janet E. Olson:** Writing – review & editing, Data curation. **Alpa V. Patel:** Writing – review & editing, Data curation. **Kathryn J. Ruddy:** Writing – review & editing, Data curation. **Dale P. Sandler:** Writing – review & editing, Data curation. **Lauren R. Teras:** Writing – review & editing, Data curation. **Clarice R. Weinberg:** Writing – review & editing, Data curation. **Jeffrey N. Weitzel:** Writing – review & editing, Data curation. **Stacey J. Winham:** Writing – review & editing, Data curation. **Siddhartha Yadav:** Writing – review & editing, Data curation. **Song Yao:** Writing – review & editing, Data curation. **Gary Zirpoli:** Writing – review & editing, Data curation. **Marketa Janatova:** Writing – review & editing, Data curation. **Zdenek Kleibl:** Writing – review & editing, Data curation. **Petra Kleiblova:** Writing – review & editing, Data curation. **Jana Soukupova:** Writing – review & editing, Data curation. **Qihong Zhao:** Writing – review & editing, Data curation. **Lisa Devereux:** Writing – review & editing, Data curation. **Paul A. James:** Writing – review & editing, Data curation. **Ian G. Campbell:** Writing – review & editing, Data curation. **Tu Nguyen-Dumont:** Writing – review & editing, Data curation. **James G. Dowty:** Writing – review & editing, Data curation. **Nadine Andrieu:** Writing – review & editing, Data curation. **Fabienne Lesueur:** Writing – review & editing, Data curation. **Dominique Stoppa-Lyonnet:** Writing – review & editing, Data curation. **Miguel de la Hoya:** Writing – review & editing, Data curation, Conceptualization. **Paolo Radice:** Writing – review & editing, Data curation. **Claus Storgaard Sørensen:** Writing – review & editing, Data curation. **Paolo Peterlongo:** Writing – review & editing, Data curation.

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