Dipeptidyl peptidase 9 triggers BRCA2 degradation by the N-degron pathway to promote DNA-damage repair

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Running title: DPP9 regulates BRCA2 stability and activity

Key words: DPP9, BRCA2, RAD51, N-degron, N-end rule, DNA damage repair

Summary

Dipeptidyl peptidase 9 (DPP9) is a serine protease cleaving N-terminal dipeptides preferentially post-proline with (patho)physiological roles in the immune system and cancer. Only few DPP9 substrates are known. Here we identify an association of human DPP9 with the tumour suppressor BRCA2, a key player in repair of DNA double-strand breaks that promotes the formation of RAD51 filaments. This interaction is triggered by DNA-damage and requires access to the DPP9 active-site. We present crystallographic structures documenting the N-terminal Met₁-Pro₂ of a BRCA2₁₋₄₀ peptide captured in the DPP9 active-site. Mechanistically, DPP9 targets BRCA2 for degradation by the N-degron pathway, and promotes RAD51 foci formation. Both processes are phenocopied by BRCA2 N-terminal truncation mutants, indicating that DPP9 regulates both stability and the cellular stoichiometric interactome of BRCA2. Consistently, DPP9-deprived cells are hypersensitive to DNA-damage. Together, we identify DPP9 as a regulator of BRCA2, providing a possible explanation for DPP9 involvement in cancer development.

Key words: DPP9, BRCA2, RAD51, Protease, degradation, N-degron pathway, N-end rule, degron, DNA damage repair, protein turnover

Introduction

Dipeptidyl peptidases 9 (DPP9) is an intracellular protease of the DPPIV family, that is critical for neonatal survival (Gall et al. 2013) with roles in the immune response (Geiss-Friedlander et al. 2009; Okondo et al. 2016; Okondo et al. 2018; Johnson et al. 2018; de Vasconcelos et al. 2019). Deregulation of DPP9 is connected to pathophysiological conditions such as tumorigenicity (Tang et al. 2017; Smebye et al. 2017; Johnson et al. 2018; Spagnuolo et al. 2013), whereby the underlying mechanisms are poorly understood.

On the molecular level, proteases of the DPPIV family are serine aminopeptidases that remove dipeptides from the N-terminus of substrates having a Pro or an Ala residue in the second position (NH₂-XaaPro/Ala) (Waumans et al. 2015). DPP8 and DPP9 are the only known intracellular members of this family, both localize to the cytosol, the nucleus and associate with the plasma membrane (Ajami et al. 2004; Justa-Schuch et al. 2014; H. Zhang, Chen, et al. 2015). DPP9 is significantly more abundant than DPP8, and its depletion leads to a drastic reduction in the capacity of cells to process proline containing peptides (Geiss-Friedlander et al. 2009). In addition to peptides, several proteins were identified as DPP9 substrates (H. Zhang, Maqsudi, et al. 2015; Wilson et al. 2013). These include the tyrosine kinase Syk, a central kinase in the B cell receptor mediated signalling pathway. Previously we showed that processing of the Syk N-terminus by DPP9 leads to Syk degradation, linking DPP9 to the N-degron pathway (Justa-Schuch et al. 2016), a branch of the ubiquitin-proteasome pathway which targets proteins with destabilizing N-terminal residues for degradation (Varshavsky 2019). Although DPP9 binds directly to Syk, in cells this interaction is stabilized by Filamin A (FLNA) (Justa-Schuch et al. 2016), a scaffold protein that localizes both to the cytoplasm and to the nucleus, where it interacts with multiple proteins thereby regulating signalling networks (Yue et al. 2013; Yue et al. 2009; Velkova et al. 2014; Yue et al. 2012).

Here, we asked whether additional FLNA partners would be substrates of DPP9. Thus, we inspected the N-terminus of known FLNA-binding proteins for a potential DPP9 cleavage sequence. This search highlighted the N-terminus of the human BRCA2 (Met-Pro-Ile), which was previously shown to bind to FLNA (Yuan & Shen 2001b). Alignment of multiple species shows that the potential DPP9 cleavage site in the N-terminus of BRCA2 is conserved in many mammals (Figure 1 - figure supplement 1). In some species, the proline is replaced by an alanine, or the isoleucine is replaced by a valine (Met-Pro/Ala-Ile/Val)

(http://slim.icr.ac.uk/proviz/proviz.php?uniprot_acc=P51587). Also these sequences present potential DPP9-cleavage sites.

BRCA2 is critical for repair of DNA double strand breaks (DSBs) by the homologous recombination (HR) pathway (Moynahan et al. 2001). Such lesions present the most harmful form of DNA damage, as they can lead to genome rearrangement and genome instability if not correctly repaired. In contrast to the repair of DSBs by the non-homologous end-joining pathway which is error prone, the repair of such breaks by HR is mostly error free. In short, the repair of DSBs by the HR pathway starts with the generation of a 3' single strand DNA (ssDNA) overhang by a process called 5' end-resection. The ssDNA is subsequently covered by protein filaments of the strand-exchange protein RAD51. These filaments form a so-called displacement loop (D-loop), which invades into homologous sequences preferentially in the sister chromatid that serves as template for repair of the lesions by DNA polymerases (Scully et al. 2019; C.-C. Chen et al. 2018). The formation of the RAD51 filament is directly regulated by BRCA2, which interacts with multiple copies of RAD51 via eight so called BRC repeats located in the centre of BRCA2 and an additional RAD51 binding domain at the C-terminus (A. A. Davies et al. 2001; Pellegrini et al. 2002; Esashi et al. 2007; O. R. Davies & Pellegrini 2007). By simultaneously binding multiple monomers of RAD51, BRCA2 provides a rapid mechanism for loading and assembly of RAD51 as filaments on the ssDNA (Thorslund et al. 2010; Jie Liu et al. 2010; Jensen et al. 2010; Carreira & Kowalczykowski 2011; Shahid et al. 2014; Sánchez et al. 2017). The importance of BRCA2 is highlighted by the fact that it is often mutated in patients with familial breast and ovarian cancers (Scully et al. 2019; C.-C. Chen et al. 2018). Intriguingly, although critical for DSB repair, BRCA2 steady-state protein levels were shown to be lower following DNA damage, a process that is counteracted by the ubiquitin-specific protease 21 (USP21) and by proteasome inhibition (Schoenfeld et al. 2004; Jinping Liu et al. 2017).

Given the interactions of DPP9 and BRCA2 with FLNA (Justa-Schuch et al. 2016; Yuan & Shen 2001a) and the presence of a conserved putative DPP9 cleavage site in an unstructured N-terminal sequence of BRCA2, we asked whether BRCA2 is regulated by DPP9. In this work we show that DPP9 promotes BRCA2 degradation and also activity. We suggest that DPP9 is critical for establishing the cellular stoichiometric interactome of BRCA2.

Results

DNA damaging conditions trigger an interaction between BRCA2 and DPP9, requiring DPP9 active site

As a starting point we asked whether DPP9 and BRCA2 interact in cells. Thus, we performed Proximity Ligation Assays (PLA), a highly sensitive method that is especially suited for detection and quantification of dynamic interactions within cells. Initial analysis of HeLa cells did not detect specific PLA signals for BRCA2-DPP9 above the background signals that are observed in control cells silenced for either DPP9 or BRCA2 (Figure 1 – figure supplement 2A and 2B). However, since BRCA2 is critical for repair of DSBs, these assays were also carried out in cells that were exposed to Mitomycin C (MMC), a chemotherapeutic agent that forms inter-strand DNA crosslinks which are converted to secondary DSBs. Strikingly, whereas MMC did not trigger a close proximity between DPP9 and BRCA2 in cells silenced for FLNA, specific signals for DPP9-BRCA2 PLA were detected both in the cytoplasm and in the nucleus of mock-silenced cells in response to MMC (Figure 1A and 1B, Figure 1 – figure supplement 2A).

Subsequently, to examine whether the interaction between DPP9 and BRCA2 involves the N-terminus of BRCA2, Surface Plasmon Resonance (SPR) assays were performed. This approach documented direct binding of purified recombinant DPP9 to a peptide corresponding to the first forty amino acids of BRCA2 (BRCA2₁₋₄₀), with a binding affinity (KD) of $16.8 \pm 0.7 \mu\text{M}$

(Figure 1C and 1D). A truncated form of the synthetic BRCA2 fragment, lacking the N-terminal residues Met₁-Pro₂ (BRCA2₃₋₄₀), bound to DPP9 with lower affinity (K_D) of 75.3±4.8 μM, highlighting the importance of the N-terminal dipeptide Met₁-Pro₂ for binding to DPP9 (Figure 1D).

Consistent with the SPR measurements, pull-down assays confirmed a direct binding between DPP9 and a construct corresponding to the first forty amino acids of BRCA2 fused to a C-terminal HA tag (BRCA2₁₋₄₀HA) (Figure 1E). These assays were also performed in the presence of SLRFLYEG or 1G244, two competitive inhibitors of DPP9 (Wu et al. 2009; Pilla et al. 2013). Previously, we had shown that binding of 1G244 or SLRFLYEG, initiates a rearrangement and a closed conformation of the DPP9 active site (Ross et al. 2018). Importantly, the binding of DPP9 to BRCA2₁₋₄₀HA was clearly blocked by SLRFLYEG and 1G244, demonstrating the importance of the open DPP9 active site conformation for binding to the BRCA2 N-terminus (Figure 1E). The significance of access to the active site of DPP9 was further challenged in cells. Consistent with the results obtained with the recombinant factors, cells treated with 1G244 did not show a significant increase in DPP9-BRCA2 PLA events in response to MMC (Figure 1F and 1G, Figure 1 – figure supplement 2C). Taken together, these data show an interaction between DPP9 and BRCA2 that is triggered by DNA damage and requires access to the DPP9 active site, a characteristic for interactions between an enzyme and its substrate.

Figure 1

Figure 1. DNA damage triggers an interaction between DPP9 and BRCA2, requiring access to DPP9 active site

(A) Proximity ligation assay (PLA) images showing that MMC triggers an interaction between endogenous DPP9 and BRCA2 in HeLa WT cells. Each white dot represents a single PLA event between DPP9 and BRCA2. Phalloidin (green) stains actin filaments, DAPI (blue) stains the nucleus. Scale bar 10 μ m.

(B) Graph summarizing independent biological replicates of the PLA for BRCA2-DPP9. In each replicate, more than 125 cells were quantified for each condition respectively. PLA of the technical control samples (NgtCntrl) did not include the BRCA2 antibody. Each point represents the mean of one replicate, normalized to signals in control HeLa cells (siNT -MMC). Mean $\pm$ SEM, data were analysed by a two-way ANOVA, with Tukey's Multiple Comparison test.

(C) Amino acid sequences of synthetic peptides corresponding to the N-terminus of intact BRCA2 (BRCA2₁₋₄₀) and truncated (BRCA2₃₋₄₀).

(D) Surface Plasmon Resonance (SPR) data showing a direct interaction of purified DPP9 with the BRCA2₁₋₄₀ peptide. Binding affinity was significantly reduced for the truncated BRCA2₃₋₄₀ peptide, which lacks the DPP9 cleavage site (Met-Pro). A serial dilution of BRCA2-derived peptides was injected over a surface covered with DPP9. Equilibrium binding isotherms obtained for interactions measured between DPP9 and BRCA2₁₋₄₀ (black line) and BRCA2₃₋₄₀ (red line). Data were fitted to a sigmoidal dose-response curve fit. Shown are mean $\pm$ SEM of the triplicate. Box summarizes the calculated affinities (KD values).

(E) Direct binding of an N-terminal BRCA2 fragment to the relaxed form of DPP9. Recombinant fragments corresponding to the N-terminus of intact BRCA2 with a C-terminal HA tag (BRCA2₁₋₄₀HA) were immobilized on HA-beads and tested for binding to purified DPP9. Binding of DPP9 to the BRCA2 N-terminal fragment was impaired in the presence of competitive DPP9 inhibitors (1G244 or SLRFLYEG). Representative data of three independent experiments is shown.

170 (F) Fewer DPP9-BRCA2 PLA interactions (white dots) in HeLa cells treated with 1G244,
171 which blocks the access to the DPP9 active site. Control cells were mock treated with DMSO.
172 Scale bar 10 μ m. Phalloidin (green) stains actin filaments, DAPI (blue) stains the nucleus.
173 (G) Graph summarizing 3 independent biological replicates of the PLA for BRCA2-DPP9. In
174 each replicate, more than 50 cells were quantified for each condition respectively. PLA of the
175 technical control samples (NgtCntrl) did not include the BRCA2 antibody. Each point
176 represents the mean of a single replicate, normalized to signals in control HeLa cells (siNT -
177 1G244). Shown are Mean $\pm$ SEM, data were analysed by a two-way ANOVA, with Tukey's
178 Multiple Comparison test.

BRCA2 N-Terminus

<i>Homo sapiens</i> (HUMAN)	MP IGSKERPTFFEIF K TRCN K -ADLGPISLNWFEELSSEAPPYNSEPAEESEHKNNNYEP	59
<i>Pan troglodytes</i> (CHIMPANZEE)	MP IGSKERPTFFEIF K TRCN K -ADLGPISLNWFEELSSEAPPYNSEPAEESEHKNNNYEP	59
<i>Mus musculus</i> (MOUSE)	MP VEYKRRPTFWEIF K ARCST-ADLGPISLNWFEELSSEAPPYNSEPPPESEYKPHGYEP	59
<i>Sus scrofa</i> (PIG)	MP IGSKERPTFFEIF K TRCNE-ADLGPVSLNWFEELSLEAPPYNSEPPSESEYKN-----	54
<i>Bos taurus</i> (BOVINE)	MP IGSKERPTFFDIF K ARC N K-ADLGPISLNWFEELSSEAPLCNSEPLEESEYKISSNET	59
<i>Felis catus</i> (CAT)	MP IGSKERPTFFEIFRTRCN K -ADLGPISLNWFEELCLEAPPYNSEPTTEESGYKISY-EP	58
<i>Canis lupus familiaris</i> (DOG)	MP VGSKERPTFFEIF K TRCNQ-ADLGPISLNWFEELSLEAPPYNSEPTTEESGYKISY-EP	58
<i>Oryctolagus cuniculus</i> (RABBIT)	MP IGSKERPTLFDIF K MRCNTVDLGPISLNWFEELSSEAPLYNSEPAEESECKINNCEP	60
<i>Rattus norvegicus</i> (RAT)	MTVEYKRRPTFWEIF K ARCST-ADLGPISLNWFEELSSEAPPYNTEPPPESEYKPPQGH	59
<i>Monodelphis domestica</i> (GRAY SHORT-TAILED OPOSSUM)	MSAGYQGKTTFEFVFKTRCSE-SDLGPISLNWFEELTSEAPPYNYEISEDPGSKTEITEV	59

Figure 1 – figure supplement 1

179
180 **Figure 1 – figure supplement 1: A potential DPP9 cleavage site in BRCA2**
181 Alignment of the BRCA2 N-terminus in several species. The potential DPP9 cleavage site
182 (Met-Pro↓Ile/Val) is shown in bold. The alignment was performed with Clustal Omega
183 (multiple sequence alignment) program. Conserved lysine residues are highlighted in green.

Figure 1 – figure supplement 2

Figure 1 – figure supplement 2: DNA damage triggers an interaction between DPP9 and BRCA2, requiring access to DPP9 active site

(A) Quantification of one PLA experiment showing close proximity between DPP9 and BRCA2 that is triggered by MMC. PLA signals from more than 125 cells were quantified in

189 each condition respectively. Mean $\pm$ SEM, data were analysed by a two-way ANOVA, with
 190 Tukey's Multiple Comparison test.

191 (B) Western Blots of the corresponding silenced cells (without MMC) analysed by PLA (A).
 192 For control, cells were silenced for DPP9 (siDPP9), BRCA2 (siBRCA2), FLNA (siFLNA) or
 193 treated with non-targeting siRNA (siNT).

194 (C) Quantification of one of three PLAs, showing that the interaction between DPP9 and
 195 BRCA2 is blocked in the presence of the inhibitor 1G244. In each experiment, signals from
 196 more than 50 cells per condition per assay were quantified. Mean $\pm$ SEM, data were analysed
 197 by a two-way ANOVA, with Tukey's Multiple Comparison test.

DPP9 removes the BRCA2 N-terminal dipeptide Met₁Pro₂

Since the interaction with BRCA2 requires access to the active site of DPP9, we next tested whether DPP9 can hydrolyse BRCA2 N-terminal peptide. *In vitro* reactions were carried out with purified DPP9 and the BRCA2₁₋₄₀ N-terminal peptide followed by mass spectrometry analysis. Control reactions contained no DPP9, or included the DPP9 inhibitor SLRFLYEG. These assays demonstrated processing of BRCA2₁₋₄₀ N-terminal peptide by purified DPP9, leading to removal of the dipeptide Met₁Pro₂, with the resulting product (BRCA2₃₋₄₀) having an isoleucine in the neo N-terminus (Figure 2A, Met₁Pro₂↓Ile₃).

To further understand the cleavage event, DPP9 crystals were soaked with the BRCA2₁₋₄₀ peptide. Strikingly, DPP9 crystals exhibited positive electron density at the active site corresponding to the N-terminal Met₁Pro₂ di-peptide with the proline residue occupying the S1 subsite. Since DPP8 and DPP9 share a very similar substrate binding mechanisms *in vitro*, and since DPP9 crystals are notorious for being less well ordered (Ross et al. 2018), we also tested for a possible interaction between DPP8 and BRCA2. Similarly, in DPP8 all 3 copies of the asymmetric unit exhibit clearly interpretable electron density for the di-peptide. The well-ordered R-segment, a hallmark of ligand binding, was well defined and ordered and supports full occupation of the active sites. There was no trace of electron density for the rest of the peptide (Figure 2B and Table 1). These structures with the di-peptide captured in the active site clearly document intra-crystalline enzymatic activity of DPP8 and DPP9 resulting in the hydrolysis of the BRCA2₁₋₄₀ N-terminal peptide, and release of the BRCA2₃₋₄₀ peptide. Together with the cellular assays, we conclude that DPP9 processes the N-terminus of BRCA2.

219

A

B

Figure 2

220

221 **Figure 2. DPP9 enzymatic activity cleaves the BRCA2₁₋₄₀ N-terminal peptide**

222 (A) A representative experiment showing in vitro processing of BRCA2₁₋₄₀ synthetic peptide

223 by purified recombinant DPP9. BRCA2₁₋₄₀ peptide was incubated with DPP9 for 6 hours.

Control samples included the DPP9 inhibitor SLRFLYEG. Samples were analysed by high-resolution liquid chromatography/tandem mass spectrometry, in quadruplicate. The panels show extracted MS1 ion chromatograms for both substrate BRCA2₁₋₄₀ peptide (MPIGSKERPT...) (labelled S, [M+5H]⁵⁺ m/z 917.8637; retention time 17.7 minutes) and product BRCA2₂₃₋₄₀ peptide (IGSKERPT...) (labelled P, [M+5H]⁵⁺ m/z 872.2451; retention time 17.6 minutes). The identity of the product peak was established both by accurate mass measurement to within 5 ppm and by product ion spectra (data not shown).

(B) Shown is the N-terminal di-peptide of BRCA2 bound to both DPP9 and to DPP8. Omit map (Fo-Fc; 3σ) of a BRCA2 peptide soaked in DPP8 crystals (C222₁) and in DPP9 crystals (P12₁1). The R-Segment and SUBA are highlighted in orange and blue, respectively. The arrow marks the position where the BRCA2 peptide is cleaved. For simplification, shown here is a monomer of DPP8. Both zoomed views are rotated 45° with respect to the monomer view to better display the ligand.

Table 1. Crystallographic parameters for DPP8 and DPP9 -BRCA2 peptide bound structures.

	DPP8 - BRCA2 peptide	DPP9 - BRCA2 peptide
Data collection		
Space group	C222 ₁	P12 ₁ 1
Resolution (Å)	44.58 - 3.20	43.47 - 3.00
	(3.28 - 3.20) ^a	(3.19 - 3.00) ^a
a, b, c (Å)	162.73 246.07 261.81	119.52 117.22 163.40
α, β, γ (°)	90, 90, 90	90, 105.74, 90
R _{meas} (%)	14.3 (139.9) ^a	15.9 (37.6) ^a

CC _{1/2} (%)	99.8 (73.6) ^a	98.9 (93.9) ^a
I/σ(I)	17.2 (1.8) ^a	5.9 (3.0) ^a
Completeness (%)	99.9 (99.2) ^a	87.8 (88.8) ^a
Multiplicity	8.37 (8.34) ^a	2.65 (2.53) ^a
Total observations	725357 (52815) ^a	202589 (33069) ^a
Total unique observations	86450 (6331) ^a	76534 (13092) ^a
Refinement		
R _{cryst} /R _{free} (%)	18.39/22.47	28.5/34.5 ^b
Number of reflections	82126 (4323) ^c	72706(3827) ^c
R.m.s.d. bond lengths (Å)	0.003	0.004
R.m.s.d. bond angles (°)	0.984	1.120
Number of atoms	20409	26316
Average B-factor (Å ²)	84.8	25.5
Ramachandran plot (%)		
Preferred region	93.4	91.9
Allowed region	5.9	7.2
Outliers	0.6	0.8
^a Values in parentheses correspond to the highest-resolution shell		
^b The relatively high values are due to the crystals displaying multiple splits (Ross et al. 2018)		
^c Values in parentheses correspond to free R-value test set (5%)		

239

240 DPP9-deficient cells are hypersensitive to genotoxic agents

241 The above data show that MMC-induced DNA damage triggers an interaction between DPP9

242 and BRCA2, which requires access to the active site of DPP9, and more importantly, that

DPP9 cleaves BRCA2 N-terminus. Since BRCA2 is critical for repair of DSBs by HR, we raised the question whether DPP9 is relevant for repair of DNA damage. A common feature for cells with defects in repair of DSBs, is a hypersensitivity to DNA-damaging agents. Thus, the response of DPP9-deficient cells to the presence of genotoxic agents was assessed by analyzing the viability of these cells and their capacity to form colonies.

To test for the importance of DPP9, we assayed HeLa DPP9 KD cells that express lower DPP9 levels (50%) due to constitutive silencing of DPP9 (Figure 3 - figure supplement 1A and 1B, cells described first in (Justa-Schuch et al. 2016)). Strikingly, cell viability assays revealed that HeLa DPP9 KD cells are significantly more sensitive to MMC compared to the corresponding HeLa WT cells (Figure 3A). Subsequently, we tested the viability of the HeLa DPP9 KD cells following exposure to Olaparib, an FDA approved-drug that is used for treatment of germline BRCA1 and BRCA2-mutated metastatic breast cancers. Olaparib is an inhibitor of poly(ADP-ribose) polymerase (PARP), which plays a role in base excision repair of single-strand DNA (ssDNA) breaks. Cells which are defective in HR are hypersensitive to Olaparib because the accumulating ssDNA breaks are converted in cells to DSBs. Notably, we find that HeLa DPP9 KD cells are significantly more sensitive to Olaparib compared to control cells (Figure 3B), thus displaying a similar pattern to previous studies showing the sensitivity of BRCA2-deficient cells to Olaparib treatment (Menear et al. 2008; Rottenberg et al. 2008; Lord & Ashworth 2016).

Furthermore, we examined the capacity of the HeLa DPP9 KD cells to form colonies following exposure to ionizing radiation (γ -radiation) as a source for DSBs. Colony formation assays revealed that HeLa DPP9 KD cells are significantly more sensitive to γ -radiation in comparison to the corresponding control cells (Figure 3C). To test for the importance of DPP9 activity we analysed the DPP9 gene knock-in mouse embryonic fibroblasts (MEFs), which express an enzymatically inactive DPP9 variant carrying a point

mutation in its catalytic serine (gki MEF DPP9^{S729A} cells were first described in (Gall et al. 2013)). Similar to the HeLa DPP9 KD cells, also the MEF DPP9^{S729A} were significantly more sensitive to γ -radiation compared to the corresponding MEF WT cells (Figure 3D).

To visualize the presence of DSBs, we followed the phosphorylation of H2AX on serine 139 (γ H2AX). This phosphorylation step is an early and decisive step in repair of DSBs, that marks the surrounding of the break and is used as a platform that initiates the recruitment of repair proteins (Turinetti & Giachino 2015). In line with the observed reduced viability, the HeLa DPP9 KD cells also accumulated significantly higher levels of γ H2AX in response to MMC (Figure 3 - figure supplement 1C and 1D). Consistently, significantly higher γ H2AX were observed in the gki MEF DPP9^{S729A} compared to the MEF WT in response to MMC (Figure 3 - figure supplement 1E and 1F). We further examined the capability of DPP9-deprived cells to recover from the DNA damage, by following the disappearance of γ H2AX. As a source for DNA damage, cells were treated with the radiomimetic antibiotic Neocarzinostatin (NCS). Following a 30 minute incubation, NCS was removed and the levels of γ H2AX foci were monitored over time. These assays revealed higher γ H2AX levels in HeLa DPP9 KD cells compared to the corresponding control cells (Figure 3E, Figure 3 - figure supplement 1G). Importantly, significantly higher γ H2AX levels were also present in gki MEF DPP9^{S729A} (Figure 3F and 3G).

Figure 3

Figure 3. Cells with reduced DPP9 activity are hypersensitive to genotoxic agents

(A and B) Dose-dependent viability assays showing a higher sensitivity of HeLa DPP9 KD cells to (A) MMC and (B) Olaparib. Mean $\pm$ SEM of three independent biological replicates,

with each experiment performed in triplicate. Data were analysed by an unpaired two-way ANOVA with Sidak's multiple comparison test.

(C and D) Quantification of colonies formed after γ -radiation of (C) HeLa WT and DPP9 KD, as well as (D) MEF WT and gki MEF DPP9^{S729A} cells, showing the mean $\pm$ SEM of the survival fraction (SF) from three independent experiments. Data were analysed by an unpaired two-way ANOVA.

(E and F) Graphs showing that (E) HeLa DPP9 KD and (F) gki MEF DPP9^{S729A} cells accumulated more γ H2AX signals in response to Neocarzinostatin (NCS) treatment compared to the corresponding control cells. All cells were treated with 250 ng/mL NCS for 30 minutes and allowed to recover for the indicated time-points. Data were analysed by an unpaired two-way ANOVA with Sidak's multiple comparison test. (E) Signals from more than 1300 cells were quantified per condition per experiment. Mean $\pm$ SEM from four independent experiments.

(F) Signals from more than 1700 cells were quantified per condition per experiment. Mean $\pm$ SEM from six independent experiments.

(G) Representative immunofluorescence images showing the accumulation and recovery of γ H2AX signals (white) in control MEF cells and in gki MEF DPP9^{S729A} cells following NCS treatment. The nucleus is shown in blue (DAPI). Scale bar 10 μ m.

Figure 3 - figure supplement 1

Figure 3 - figure supplement 1: high γ H2AX levels in DPP9-deprived cells

(A) Western-blot showing that the protein levels of DPP9 in HeLa DPP9 KD cells are significantly lower than those in the corresponding HeLa WT cells. DPP9 protein levels are not altered following exposure to 300 nM MMC for 24 hours. Tubulin was analysed as loading control. Representative images of one from three independent experiments are shown.

(B) Quantification of DPP9 signals in HeLa WT and DPP9 KD cells following exposure to 300 nM MMC for 24 hours. Tubulin was analysed as loading control. Mean $\pm$ SEM. Data were analysed by a two-way ANOVA, with Tukey's Multiple Comparison test.

(C) Higher γ H2AX signals in HeLa DPP9 KD cells following exposure to 300 nM MMC for 24 hours. Tubulin was analysed as loading control.

(D) Quantification of the γ H2AX signals in HeLa DPP9 KD cells and HeLa WT cells described in (C). Mean $\pm$ SEM. Data were analysed by a two-way ANOVA, with Tukey's Multiple Comparison test.

(E) Higher γ H2AX signals in gki MEF DPP9^{S729A} cells expressing enzymatically inactive DPP9 compared to MEF WT control cells following exposure to 300 nM MMC for 24 hours. Tubulin was analysed as loading control.

(F) Quantification of the γ H2AX signals in gki MEF DPP9^{S729A} cells and MEF WT cells described in (E). Mean $\pm$ SEM. Data were analysed by a two-way ANOVA, with Tukey's Multiple Comparison test.

(G) Accumulation and reduction of γ H2AX signals (white) in the nuclei of HeLa DPP9 KD and HeLa WT cells following treatment with 250 ng/mL NCS for 30 minutes. Cells were allowed to recover for the specified time-points and then fixed. Shown are representative images of one from three independent experiments. γ H2AX is shown in white, the nucleus is shown in blue. Scale bar 10 μ m.

DPP9 targets BRCA2 for degradation following DNA damage

The results above show that cells-deprived of DPP9 are hypersensitive to genotoxic agents and accumulate more DSBs, indicating that the activity of DPP9 is critical for the capacity of cells to survive in response to DSBs. Since DPP9 interacts and cleaves BRCA2 N-terminus, we inspected more closely the effect of DPP9 on BRCA2. As a first step, we analysed for the interaction of BRCA2 with the Partner and localizer of BRCA2 (PALB2), a protein that ensures the correct intra-nuclear localization of BRCA2 following DNA-damage (Xia et al. 2006; F. Zhang et al. 2009; Oliver et al. 2009).

In line with published data, we detected PLA signals documenting interactions between endogenous BRCA2 and PALB2 (Figure 4A and 4B, Figure 4 - figure supplement 1). Strikingly, significantly more BRCA2-PALB2 PLA events were present in cells silenced for DPP9, in the presence of MMC (Figure 4A and 4B, Figure 4 - figure supplement 1), suggesting that DPP9 limits the BRCA2-PALB2 interaction. Furthermore, we observed slightly higher steady state levels of BRCA2 in cells silenced for DPP9, raising the question whether DPP9 influences BRCA2 stability (Figure 4 - figure supplement 1).

To address this question, we performed cycloheximide (CHX) chase assays. These demonstrated that endogenous BRCA2 is relatively stable with a half-life greater than 8 hours, and that the addition of MMC stimulates an accelerated turnover of BRCA2, displaying a considerably shorter half-life of 2-3 hours, while the stability of RAD51 was unaltered (Figure 4C and 4D, Figure 4 - figure supplement 2A and 2B). The rapid MMC-induced degradation of BRCA2 was less pronounced in the presence of the proteasome inhibitor MG132, pointing to a role of the ubiquitin-proteasome pathway (Figure 4 - figure supplement 2C and 2D). CHX chase assays were also conducted in the DPP9 KD cells, revealing that while the half-life of RAD51 was not affected, the MMC-triggered degradation of BRCA2 was significantly less pronounced in the HeLa DPP9 KD cells compared to HeLa wild-type cells (Figure 4E and 4F,

Figure 4 - figure supplement 2E and 2F). A similar stabilization of BRCA2 was observed in cells silenced for DPP9 (Figure 4G and 4H).

Next we asked whether BRCA2 stability is indeed mediated by the N-terminal dipeptide Met₁-Pro₂, which we showed to be removed by DPP9 (Figure 2). To this end, a BRCA2ΔMP₍₃₋₁₀₀₀₎ mutant lacking these two residues was cloned to mimic a BRCA2 product which would be generated following cleavage by DPP9 (MP↓IGSK → IGSK) (Figure 4 - figure supplement 2G). To produce the required N-terminus (without the initiator methionine) an N-terminal ubiquitin tag was fused to the BRCA2 constructs. This technique relies on endogenous ubiquitin proteases that remove the ubiquitin thereby exposing the desired N-terminus (Varshavsky 2005). CHX chase assays show that the variant containing the unmodified N-terminus BRCA2₍₁₋₁₀₀₀₎ was relatively stable with a half-life greater than 8 hours, similar to that of the endogenous BRCA2 in the absence of MMC. On the other hand, BRCA2ΔMP₍₃₋₁₀₀₀₎, was significantly less stable, with a half-life of 2-3 hours (Figure 4I and 4J), similar to the turnover rate of BRCA2 following MMC exposure.

Taken together, we show that DPP9 limits the interaction of BRCA2 with PALB2 and targets BRCA2 for degradation. DPP9-mediated destabilization of BRCA2 is triggered by the presence of DNA-damage, and is mimicked by the BRCA2ΔMP₍₃₋₁₀₀₀₎ truncation mutant.

Figure 4

Figure 4. DPP9 triggers BRCA2 degradation in response to MMC

(A) PLA quantification showing more BRCA2-PALB2 events in HeLa cells silenced for DPP9 compared to cells treated with non-targeting siRNA (siNT). The graph summarizes five independent biological replicates. In each replicate, more than 100 cells were quantified for

each condition respectively. PLA of the technical control samples (NgtCntrl) did not include the BRCA2 antibody. Each point represents the mean of one replicate, normalized to signals in control HeLa cells (siNT -MMC). Shown are Mean $\pm$ SEM, data were analysed by a two-way ANOVA, with Tukey's Multiple Comparison test.

(B) Representative PLA images described in (A), showing more BRCA2-PALB2 events (white) in HeLa cells silenced for DPP9 compared to cells treated with non-targeting siRNA (siNT). Actin (Phalloidin) is shown in green and the nucleus (DAPI) in blue. Scale bar 10 μ m.

(C) Cycloheximide (CHX)-chase assays in HeLa WT cells, showing a rapid turnover of endogenous BRCA2 following exposure to 300 nM MMC (24 hours). Vinculin was analysed as a loading control.

(D) Graph summarizing three independent CHX-chase assays performed as described in (C). The ratios of BRCA2 to Vinculin were calculated using 100% at time 0 hours. Mean $\pm$ SEM, paired two-tailed t-test.

(E) CHX-chase assays in the presence of MMC showing that BRCA2 is more stable in HeLa DPP9 KD cells than in the corresponding HeLa WT cells.

(F) Graph summarizing four independent CHX-chase assays performed as shown in (E). Mean $\pm$ SEM, paired two-tailed t-test.

(G) CHX-chase of HeLa WT cells in the presence of MMC, revealing a greater stability of BRCA2 in DPP9 silenced cells compared to cells treated with non-targeting siRNA oligonucleotides.

(H) Graph summarizing three independent CHX-chase assays performed as shown in (G). Mean $\pm$ SEM, paired two-tailed t-test.

(I) HeLa WT cells were transfected with BRCA2₁₋₁₀₀₀ and BRCA2 Δ MP₃₋₁₀₀₀ constructs (both tagged at the carboxy terminus with FLAG) and co-transfected with a GFP expressing plasmid, as transfection and loading control. Graph summarizing three independent CHX-chase assays

performed in HeLa WT cells transfected with BRCA2₁₋₁₀₀₀ and BRCA2ΔMP₃₋₁₀₀₀-FLAG constructs. BRCA2-FLAG signals are related to the transfection and loading control GFP. BRCA2 level in relation to GFP were defined as 100 % at time 0 hours. Mean ± SEM, paired two-tailed t-test.

(J) Representative Western Blots of transfected cells described in (I). Control cells were transfected with GFP only (U.T).

Figure 4 - figure supplement 1

Figure 4 - figure supplement 1: more BRCA2-PALB2 events in HeLa cells silenced for DPP9

PLA quantification showing more BRCA2-PALB2 events in HeLa cells silenced for DPP9 compared to cells treated with non-targeting siRNA (siNT). PLA of the technical control samples (NgtCntrl) did not include the BRCA2 antibody. Actin (Phalloidin) is shown in green and the nucleus (DAPI) in blue. Shown is one representative of 4 independent experiments. Mean ± SEM, data were analysed by a two-way ANOVA with Tukey's Multiple Comparison test. Western blot showing steady state protein levels of these samples.

Figure 4 - figure supplement 2

Figure 4 - figure supplement 2: RAD51 turnover is not affected by DPP9

(A) Cycloheximide (CHX)-chase assays in HeLa WT cells, showing that the turnover of endogenous RAD51 is unaltered by exposure to MMC (300 nM, 24 h). Tubulin was analyzed as a loading control.

(B) Graph summarizing three independent CHX-chase assays performed as described in (A). The ratio of RAD51 to tubulin were defined as 100% at time 0 hours. Mean $\pm$ SEM, paired t-test.

(C) CHX-chase of HeLa WT cells in the presence of MMC, showing that BRCA2 is more stable in cells treated with the proteasome inhibitor MG132 (100 μ M).

(D) Graph summarizing three independent CHX-chase assays performed as described in (C). Mean $\pm$ SEM, paired t-test.

(E) CHX-chase of HeLa WT and HeLa DPP9 KD cells in the presence of MMC, showing that RAD51 turnover is not affected by DPP9.

(F) Graph summarizing three independent CHX-chase assays performed as described in (E). Mean $\pm$ SEM, paired t-test.

(G) Graphical presentation of the plasmids transfected in (E and F).

DPP9 activity promotes RAD51 filament formation

Our findings show that although DPP9 triggers the degradation of BRCA2 following DNA damage, DPP9 activity is critical for cell survival under these conditions. The main role of BRCA2 in repair of DSB is to promote the assembly of RAD51 filaments on the ssDNA overhangs (Thorslund et al. 2010; Jensen et al. 2010; Jie Liu et al. 2010; Jasin & Rothstein 2013). Thus, we aimed to better understand these seemingly conflicting observations, by examining the effect of DPP9 on RAD51, the downstream interactor of BRCA2.

Pulse chase experiments have revealed that in contrast to BRCA2, the turnover of RAD51 is not altered in DPP9-deprived cells (Figure 4 - figure supplement 2E and 2F), suggesting that the effect of DPP9 is specific for BRCA2. However, fractionation assays showed that less RAD51 is present in chromatin fractions from HeLa DPP9 KD cells compared to those from HeLa WT cells (Figure 5A). Furthermore, cells silenced for DPP9 did not display RAD51 foci

(RAD51 filaments) in response to MMC (Figure 5B and 5C, Figure 5 - figure supplement 1A and 1B). Consistently, fewer RAD51 foci were observed in the gki MEF DPP9^{S729A} cells in response to MMC, in comparison to the MEF WT cells (Figure 5D and 5E, Figure 5 - figure supplement 1C), strongly suggesting that the enzymatic activity of DPP9 is necessary to promote the assembly of RAD51 filaments.

Finally, we asked whether the reduced formation of RAD51 foci in DPP9-deprived cells is caused by the processing of BRCA2 N-terminus. More specifically we asked what is the role of the dipeptide Met₁-Pro₂ in BRCA2 N-terminus. Thus, we compared the capacity of full length BRCA2 (BRCA2₁₋₃₄₁₈) and a BRCA2ΔMP truncation variant (BRCA2ΔMP₃₋₃₄₁₈) to recover the RAD51 foci formation in BRCA2-silenced cells (Figure 5F and – 5G, Figure 5 - figure supplement 1D-F). To prevent the processing of the full length BRCA2 (BRCA2₁₋₃₄₁₈) by DPP9, both constructs were transfected into HeLa DPP9 KD cells. A silent mutation was introduced into both plasmids to acquire resistance to the BRCA2 siRNA. In line with the pulse chase experiments revealing that BRCA2 mutants lacking the N-terminal Met₁-Pro₂ are less stable (Figure 4I and J), BRCA2ΔMP₃₋₃₄₁₈ protein levels were lower than that of the WT BRCA2₁₋₃₄₁₈ variant (Figure 5 - figure supplement 1E), although the same amount of plasmid was transfected. Nonetheless, a significant increase in the RAD51 foci was measured in cells expressing BRCA2ΔMP₃₋₃₄₁₈ but not in those expressing WT BRCA2₁₋₃₄₁₈ (Figure 5F and 5G, Figure 5 - figure supplement 1E and 1F). Taken together these results strongly suggest that DPP9 regulates RAD51 filament formation by processing the N-terminus of BRCA2.

Figure 5

Figure 5. Silencing of DPP9 disrupts RAD51 foci formation

(A) Less RAD51 is associated with chromatin following exposure to MMC in HeLa DPP9 KD cells compared to HeLa WT cells. Histone 3 (H3) was assayed as loading control. Shown is a representative Western Blot of three independent experiments.

(B) Graph summarizing four independent biological replicates of RAD51 showing that fewer RAD51 foci are present in response to MMC in cells silenced for DPP9 compared to HeLa cells targeted with non-coding siRNA (siNT). In each replicate, more than 60 cells were quantified for each condition respectively. Each point represents the mean of one biological replicate, normalized to signals in untreated HeLa cells (siNT-MMC). Shown are Mean $\pm$ SEM, data were analysed by a two-way ANOVA with Tukey's Multiple Comparison test.

(C) Representative immunofluorescence images of RAD51 as described in (B). Control cells were silenced for BRCA2 or RAD51. The nucleus (DAPI) is shown in blue, RAD51 in white. Scale bar 10 μ m.

(D) Graph summarizing three independent biological replicates of RAD51 showing fewer MMC-triggered RAD51 foci in gki MEF DPP9^{S729A} compared to MEF WT cells. Each dot represents the mean of one biological replicate, normalized to signals in MEF WT cells – MMC. In each replicate, more than 60 cells were quantified for each condition respectively. Mean $\pm$ SEM, data were analysed by a two-way ANOVA with Tukey's Multiple Comparison test.

(E) Immunofluorescence images revealing fewer RAD51 foci (white) in gki MEF DPP9^{S729A} cells expressing inactive DPP9 compared to the MEF WT cells following exposure to MMC. The nucleus (DAPI) is shown in blue, RAD51 in white. Scale bar 10 μ m.

(F) Significantly more RAD51 foci are present in cells transfected BRCA2 Δ MP (3-3418) compared to BRCA2 silenced cells or cells expressing the untruncated BRCA2. HeLa DPP9 KD cells were silenced for BRCA2 and transfected with BRCA2 Δ MP₍₃₋₃₄₁₈₎ or BRCA2₍₁₋₃₄₁₈₎ with the untruncated N-terminus. The graph summarises the results from four independent

504 biological replicates. In each replicate, more than 60 cells were quantified for each condition
 505 respectively. Each point represents the mean number of RAD51 foci of one biological replicate,
 506 normalized to signals in HeLa DPP9 KD cells silenced for BRCA2. Shown are Mean $\pm$ SEM,
 507 data were analysed by a one-way ANOVA.

508 (H) Representative immunofluorescence images of cells described in (G) showing the
 509 formation of RAD51 foci in HeLa DPP9 KD cells transfected with full-length BRCA2 (1-
 510 3418) or BRCA2 Δ MP (3-3418), following exposure to MMC. The nucleus (DAPI) is shown
 511 in blue, RAD51 in white. Scale bar 10 μ m.

Figure 5 – figure supplement 1

Figure 5 - figure supplement 1: Silencing of DPP9 disrupts RAD51 foci formation

(A) Fewer RAD51 foci are formed in response to MMC in HeLa cells silenced for DPP9, compared to control cells silenced with non-targeting siRNA. Shown is a representative quantification of one biological replicate. Signals of more than 60 cells were quantified for each condition respectively. Mean $\pm$ SEM, data were analysed by a two-way ANOVA with Tukey's Multiple Comparison test. Representative immunofluorescence images of this experiment are shown in Figure 5C.

(B) Western Blot showing the protein levels of the corresponding silenced cells used for the immunofluorescence shown in (A).

(C) Shown is a representative quantification of one biological replicate showing fewer RAD51 foci in gki MEF DPP9^{S729A} compared to MEF WT cells. Per experiment, signals of more than 50 cells were quantified for each condition respectively. Mean $\pm$ SEM, data were analysed by a two-way ANOVA with Tukey's Multiple Comparison test.

(D) Graphical presentation of the two constructs transfected into HeLa DPP9 KD cells, silenced for BRCA2 (E) and (F) and the graphical representation of the silencing and transfection scheme.

(E) Western Blot of HeLa DPP9 KD cells that were silenced for endogenous BRCA2 (siBRCA2) and transfected with full-length BRCA2 (1-3418) or with a BRCA2 Δ MP (3-3418) construct. Both constructs were fused to an N-terminal ubiquitin tag, which is removed in the cells to expose the desired N-terminus.

(F) Significantly more RAD51 foci are present in cells transfected BRCA2 Δ MP₍₃₋₃₄₁₈₎ compared to BRCA2 silenced cells or cells expressing the untruncated BRCA2. Shown is a representative quantification of one biological replicate. HeLa DPP9 KD cells that were silenced for endogenous BRCA2 (siBRCA2) and transfected with full-length BRCA2₍₁₋₃₄₁₈₎ or with a BRCA2 Δ MP₍₃₋₃₄₁₈₎ construct. Both constructs were fused to an N-terminal ubiquitin tag, which is removed in the cells to expose the desired N-terminus (D)

Discussion

Here we report an unexpected role for DPP9 activity in promoting DNA damage repair. We show that DPP9-deprived cells are hypersensitive to genotoxic agents, accumulate higher γ H2AX levels, and show fewer RAD51 foci. Moreover, we identify a previously undescribed interaction between the tumour suppressor BRCA2 and DPP9, which in cells requires access to DPP9 active site. Mass spectrometry analysis shows cleavage and removal of the N-terminal dipeptide Met₁-Pro₂ from the BRCA2₁₋₄₀ peptide. Moreover, crystallographic structures of DPP9 crystals soaked with the BRCA2₁₋₄₀ peptide, reveal the dipeptide Met₁-Pro₂ of BRCA2 captured in the active site of DPP9, demonstrating intra-crystalline enzymatic activity.

DPP9 targets BRCA2 to the N-degron pathway

Functionally, DPP9 determines the turnover of BRCA2. Regulated protein degradation is an essential response for maintenance of cellular homeostasis. It is important for removal of mutated or damaged proteins and to establish a correct protein stoichiometry. In this context, BRCA2 undergoes degradation in response to mild hyperthermia (Krawczyk et al. 2011), and upon silencing of the BRCA2-binding protein DSS1 (Li et al. 2006), conditions which could lead to protein aggregation.

BRCA2 protein levels are also known to be lower following DNA damage (Schoenfeld et al. 2004; Jinping Liu et al. 2017). CHX chase assays presented here confirm that DNA damage triggers an accelerated turnover of BRCA2, a process requiring the proteasome. More importantly, we show that DPP9, which cleaves only two residues from the N-termini of its substrates, initiates the MMC-mediated degradation of BRCA2, a protein of 384 kDa. Furthermore, this accelerated turnover is mimicked by a BRCA2 truncation mutant lacking the N-terminal Met₁-Pro₂ dipeptide.

564

565 The degradation of proteins based on the sequence of their N-terminus is known as the N-
 566 degron pathway, and in eukaryotes requires the ubiquitin-proteasome pathway (Varshavsky
 567 2019). Previously we showed that by trimming the N-terminus of Syk (Met-Ala), DPP9 targets
 568 the kinase to the N-degron pathway (Justa-Schuch et al. 2016). The results presented here show
 569 that similarly, the removal of the N-terminal Met₁-Pro₂ dipeptide, converts BRCA2 to an
 570 unstable protein, demonstrating BRCA2 to be an N-degron substrate. Notably, N-terminal
 571 proline residues are characteristic of extremely poor N-degron substrates (Bachmair et al.
 572 1986). An exception are gluconeogenic enzymes with N-terminal prolines that are highly
 573 stable, but targeted for the N-degron pathway by a class of E3 ligases that specifically
 574 ubiquitinate these enzymes upon transition of yeast to a glucose-containing medium (S.-J. Chen
 575 et al. 2017; Dong et al. 2018; Melnykov et al. 2019). Here, we add DPP9 as an alternative route
 576 that cells apply to target proteins with a stabilizing N-terminal proline, by converting these to
 577 N-degrons. Similar to the induced degradation of the gluconeogenic enzymes by the N-degron
 578 pathway, also the degradation of BRCA2 is not constitutive, but instead triggered by DNA
 579 damage.

580

581 DPP9 activity regulates RAD51 foci formation

582 Here we show that although it accelerates the turnover of BRCA2 in response to DNA damage,
 583 DPP9 promotes RAD51 foci formation, a process mimicked by the BRCA2 Δ MP mutants.
 584 These observations suggest that cleavage of BRCA2 N-terminus by DPP9 may have a dual
 585 function: activation of BRCA2 in addition to BRCA2 degradation. While we cannot exclude
 586 that BRCA2 Δ MP is more active compared to BRCA2 with the unprocessed N-terminus, this
 587 scenario is not highly likely since the BRC repeats which mediate the interactions of BRCA2
 588 with several RAD51 copies are located to the centre of BRCA2, and an additional RAD51

binding domain locates to the C-terminus (A. A. Davies et al. 2001; Pellegrini et al. 2002; Esashi et al. 2007; O. R. Davies & Pellegrini 2007).

An alternative possible explanation is that the DPP9-mediated degradation of BRCA2 may positively regulate DNA repair. Turnover of key DNA repair proteins is emerging as a general concept. For example, the SUMO-targeted ubiquitin E3 ligase RNF4 is required for DSBs repair by targeting MDC1 and RPA1 at DNA-damage sites (Galanty et al. 2012; Luo et al. 2012; Yin et al. 2012). Similarly, the ubiquitin E3 ligase RFWD3, facilitates the HR repair by ubiquitinating RAD51 and RPA, leading to the removal of both proteins from DSB sites (Gong & J. Chen 2011; Shangfeng Liu et al. 2011; Elia et al. 2015; Inano et al. 2017; Feeney et al. 2017). However, the majority of the BRCA2-DPP9 PLA events are cytosolic, suggesting that the DPP9-mediated turnover of BRCA2 may play a role other than its removal from the DSBs. Several publications have previously shown that BRCA2 promotes RAD51 filament assembly by binding simultaneously to several molecules of RAD51 and that maintenance of the sub-stoichiometric ratio of BRCA2 compared to that of RAD51 is critical for filament formation (Yang et al. 2005; Thorslund et al. 2010; Jie Liu et al. 2010; Jensen et al. 2010; Shahid et al. 2014). The importance of a lower BRCA2 concentration relative to that of RAD51 was also documented in cellular assays (Magwood et al. 2012). Thus, DPP9-mediated degradation of BRCA2 following DNA damage, may promote RAD51 filament formation by reducing the relative cellular concentration of BRCA2, while those of RAD51 remain unaltered (Figure 6). Of note, similar to DPP9, also USP21 acts as a positive mediator of BRCA2. In contrast to DPP9, USP21 deubiquitinates BRCA2 to increase BRCA2 stability (Jinping Liu et al. 2017). Taken together with the data presented here, we suggest that BRCA2 steady state levels are tightly regulated and respond quickly to changing cellular conditions. Thus, the amount of BRCA2 in response to DNA-damage is determined by DPP9, but can be fine-tuned through

the down-stream deubiquitinating enzyme USP21. How DNA damage signals for DPP9 to process BRCA2 will be addressed in future work.

Outlook

Carriers of inherited mutations in BRCA2 have a high risk (> 50 %) to develop breast cancer (Mavaddat et al. 2013). Since DPP9 regulates BRCA2, we postulate that deregulation of DPP9 may lead to accumulation of unrepaired DSBs which could lead to cancer development. In this respect, data from The Human Protein Atlas show a correlation between low DPP9 mRNA expression and poor overall survival for patients with breast cancer compared to individuals with tumors displaying higher DPP9 expression who showed a better overall survival (Figure 6 - figure supplement 1) (Human Protein Atlas, (Justa-Schuch et al. 2016)). Such a correlation is not observed for its close homolog DPP8.

Interestingly, DPP9 fusion genes which lead to a premature stop codon have been identified in patients with a high-grade serous ovarian carcinoma, probably leading to lower DPP9 protein expression (Smebye et al. 2017). Such DPP9-fusions mimic the situation in DPP9 KD and gki DPP9^{S729A} MEF cells thus, we predict that defects in BRCA2 interactions and degradation in these patients, resulting in accumulation of unrepaired DSBs. Finally, cells expressing lower DPP9 levels display a high sensitivity to MMC, γ radiation and Olaparib. Thus, DPP9 inhibition in combination with for example Olaparib or radiation, may represent future potential therapies for breast-cancer.

Figure 6

Figure 6. Model: DPP9 promotes DNA damage repair by triggering the degradation of BRCA2. MMC triggers the trimming of BRCA2 N-terminus by DPP9 resulting in its degradation by the N-degron pathway, while RAD51 remains stable. This reduction of BRCA2 protein levels changes the ratio of RAD51 to BRCA2. A high RAD51 and lower BRCA2 stoichiometry has been shown to be beneficial for the formation of RAD51 filaments.

Figure 6 – figure supplement 1: Low DPP9 mRNA expression correlate with poor overall survival for patients with breast cancer

644 Graphs show separation of Breast cancer patients from The Human Protein Atlas into “high
 645 DPP9 or DPP8” (n=282, 445) and “low DPP9 or DPP8” (n =408, 630) mRNA expression
 646 (greater than or less than 9.678 or 4.36 reads per kilobase per million, respectively). Results
 647 are displayed in Kaplan-Meier survival plots; p values were calculated by log-rank (Mantel-
 648 Cox). These revealed a better overall survival in patients with higher levels of DPP9
 649 expression, while differences in the DPP8 levels were not statistically significant.

Declaration of Interests

The authors declare no competing interests

Materials and Methods

Key Resources Tables

Table 2: Antibodies

Antibodies	Method	Source	Identifier
BRCA2 Mouse	IF/PLA	R&D	MAB2476
BRCA2 Rabbit	IF/PLA	Santa Cruz	sc-8326
BRCA 2 Rabbit	WB	Abcam	123491
DPP9 Rabbit	WB	Abcam	AB42080
DPP9 Goat	IF/PLA	Self-made	(Justa-Schuch et al. 2016)
Filamin A Rabbit	WB	Novus	nb-100-58812
Filamin A Mouse	IF/PLA	Millipore	MAB1680
H2AX-P Rabbit	WB	Cell Signaling	#9718
H2AX-P Mouse	IF	Millipore	#05-636
H3 Rabbit	WB	Cell Signaling	#9715
PALB2 Rabbit	IF/PLA	Bethyl/biomol	A301-246M
Phalloidin 488	IF/PLA	Abcam	Ab176753
RAD51 Rabbit	IF/WB	Abcam	ab63801
Tubulin Mouse	WB	Santa Cruz	sc-32293
Vinculin Mouse	WB	Sigma	V9131

Table 3: Chemicals

Chemical	Source	Identifier
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1G244 (in DMSO)	AK Scientific, Inc.	#Y0432
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	#G7571
Cycloheximide (CHX)	Sigma	#C7698
Lipofectamine RNAimax	Thermo Fisher	#13778150
Mayer's Hemalum	Merck	#1.09249.0500
Mitomycin C (MMC)	Sigma	#M4287
MG132 (in DMSO)	Sigma	#C2211
Neocarzinostatin (NCS)	Sigma	#N9162
Olaparib	Selleck Chemicals	#AZD2281
Puromycin	Sigma	#P8833
Aprotinin	Carl ROTH	A162.3
Leupeptin	Carl ROTH	CN33.4
Pepstatin	Carl ROTH	2936.3
Pierce Anti-HA magnetic beads	Thermo Scientific	#88836
Ni-NTA Agarose	Qiagen	#1018244
DuoLink in Situ PLA probe Mouse plus	Sigma	#DUO92001
DuoLink in Situ PLA probe Rabbit plus	Sigma	#DUO92002
DuoLink in Situ PLA probe Mouse minus	Sigma	#DUO92004
DuoLink in Situ PLA probe Goat minus	Sigma	#DUO92006
DuoLink in Situ Detection Reagents Red	Sigma	#DUO92008
Phusion DNA polymerase	NEB	#M0363S

659

660 **Table 4: siRNA oligonucleotides**

Oligo	SiRNA sequence	Source	Identifier
DPP9	GGAUCAAUGUUCAUGACAUTT	Invitrogen	10620310
DPP9	GCCACCAGGUUUAUCCAATT	Invitrogen	10620310
BRCA2	GAAGAAUGCAGGUUUAUAUA	Dharmacon	D-003462-04-0050
FLNA	GCAGGAGGCUGGCGAGUAU	Dharmacon	J-012579-05-0050
FLNA	GCAGGAGGCUGGCGAGUAU	Dharmacon	J-012579-05-0050

Table 5: Primers

Oligo	sequence	Source	
BRCA2 fwd	GAGGCTCCGCGGTGGAATGCCTATTGGATCCAA AGAGAGGCCAACATTT	SIGMA	BRCA2 ₁₋₁₀₀
BRCA2 ΔMP fwd	GAGGCTCCGCGGTGGAATTGGATCCAAAGAGAG GCCAACATTT	SIGMA	BRCA2 ₃₋₁₀₀
BRCA2 FLAG rev	TTGCTCATGCGCCGCTCTTGTCGTCATCGTCTT TGTAAGTCAATTGGACCTAAGAGTCCTGCCC	SIGMA	BRCA2 ₁₋₁₀₀ , BRCA2 ₃₋₁₀₀
SUMO1-BRCA2 fwd	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGA TATACATATGTCTGACCAGGAGGCAAAAC	SIGMA	BRCA2 ₁₋₄₀
SUMO1-BRCA2 rev	TTTCAAAAAATGTTGGCCTCTCTTTGGATCCAAT AGGCATACCCCCCGTTTGTTCCTG	SIGMA	BRCA2 ₁₋₄₀

Table 6: Peptides

Peptide	Sequence	Purity	Source
BRCA2 ₁₋₄₀	MPIGSKERPTFFEIFKTRCNKADLGPISLWFEELSSEAP	>85 %	Genescript
BRCA2 ₃₋₄₀	IGSKERPTFFEIFKTRCNKADLGPISLWFEELSSEAP	>85 %	Genescript
SLRFLYEG	SLRFLYEG	>85 %	Genescript

Table 7: Software

Software	Source
DuoLink software	Sigma
ImageStudio/ ImageStudio Lite 4.0.21	LI-COR
LSM 510 Release Version 4.0 SP2	Zeiss
LSM Image Browser	Zeiss
Celigo Software	Celigo
NanoDrop 2000 Software	ThermoScientific
Prism 8	Graphpad

METHOD DETAILS

Cell culture

HeLa DPP9 stable knock down cells (DPP9 KD) and the corresponding HeLa WT cells (Genescript) (Justa-Schuch et al. 2016) were cultured at 37°C and 5 % CO₂ in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. To maintain the selection pressure on the DPP9 KD cells, 1.5 µg/ml puromycin (Sigma-Aldrich, Germany) was added to the growth medium. MEF wt and gkiMEF DPP9^{S729A} (Gall et al. 2013) cells were cultured at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cloning of the BRCA2 fusion vectors

BRCA2 construct covering the first 1000 amino acids (BRCA2₁₋₁₀₀₀) and a corresponding BRCA2 lacking the N-terminal dipeptide Met-Pro (BRCA2₃₋₁₀₀₀) were cloned into pcDNA3.1+ vector with a Ubiquitin tag fused at the N-terminus to provide a defined N-terminus, as first described by Bachmair and Varshavsky (Bachmair et al. 1986). As template we used pcDNA3 236HSC WT BRCA2, a kind gift from Mien-Chie Hung (Wang et al. 2002) obtained from Addgene. SUMO1-BRCA2₁₋₄₀-HA-His constructs were cloned by adding SUMO1 to the N-terminus of BRCA2₁₋₃₉-3HA-His pET11a (custom-made from Genescript), using the Gibson Chew Back and Anneal Assembly (CBA) as described in (Torella et al. 2014). Primers are listed in Table 4. The presence of the inserts was verified by restriction, followed by sequencing.

Silencing of HeLa cells

For silencing of DPP9, HeLa cells were transfected with lipofectamine RNAi max (Cat #13778150) following the manufacturer's instructions. Cells were analysed 72 hours after

transfection. For MMC treatment, cells were treated with MMC 48 h after silencing and harvested 24 hours later with Laemmli Sample Buffer (0,1 M Tris-HCl pH 6.8, 4% SDS, 20 % glycerol, 0.02% bromophenol blue, 100 mM DTT). Alternatively, cells were fixed on coverslips with 4 % formaldehyde as described below.

Protein purification

Human recombinant DPP9 was expressed from Sf9 cells and purified essentially as described in (Pilla et al. 2012). For purification of N-terminally His-HA-tagged fragments corresponding to the N-terminus of BRCA2 (BRCA2₁₋₄₀HA-His), SUMO1BRCA2₁₋₄₀HA-His was transformed into BL21 (DE3 codon+) cells and induced by 1 mM IPTG for 2 h at 30 °C. Cells were lysed in 25 ml Lysis Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazol pH 8.0, 1 mM β-Mercaptoethanol and protease inhibitors: 1 μg/ml Aprotinin, 1 μg/ml Leupeptin and 1 μg/ml Pepstatin), by sonification. The 100,000 g supernatant was bound to Ni-NTA Agarose (Qiagen) for 2 h. Beads were thoroughly washed with Lysis buffer lacking protease inhibitors and incubated with 20 μg SENP over night to release SUMO1. Following extensive washing with Lysis buffer, BRCA2₁₋₄₀HA-His was eluted with Elution buffer (50 mM Na-Phosphat pH 8.0, 300 mM NaCl, 250 mM Imidazol, 1 mM β-Mercaptoethanol).

Pull-down assay

To test for an interaction between recombinant DPP9 and a BRCA2 N-terminal fragment, BRCA2₁₋₄₀HA-His (2 μg) was incubated with magnetic anti-HA beads (Pierce Anti-HA magnetic beads, Cat# 88836, Thermo Scientific) in a Binding buffer containing (25mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.01 % (v/v) IGEPAL, 1 mM DTT), for 1 hour at 4 °C. Next recombinant DPP9 (2 μg) was added and incubated over-night at 4°C. Where stated, DPP9 was preincubated for 1 hour with the following inhibitors: 100 μM 1G244 or 110 μM

SLRFYEG. Following extensive washing, with the same buffer, bound proteins were eluted with the HA peptides and analysed by Western Blot.

Surface plasmon resonance (SPR)

Direct interactions between recombinant DPP9 and BRCA2-derived N-terminal peptides (1-40 and 3-40 amino acids) were analysed employing surface plasmon resonance (SPR). The surface of a NiHC1000m sensorchip (Xantec Bioanalytics, Duesseldorf, Germany) was rinsed with 0.5 M EDTA (pH 8.0) solution and afterwards conditioned in SPR running buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 50 μ M EDTA, 0.005 % TWEEN-20). The chip-surface was loaded with Ni²⁺-ions, injecting 300 mM NiSO₄ solution. A 200 nM solution of His-tagged DPP9 (ligand) was injected over the chip surface and immobilized to a surface density of 800-1000 μ RIU (Refractive index units; response observed), at a flow rate of 15 μ l/min. A serial dilution of the analyte (BRCA2 peptides) diluted in SPR running buffer was injected, and association was followed for 4.5 min. Dissociation of the analyte was monitored for 7 minutes. For each analyte concentration, the surface was regenerated, injecting 0.5 M EDTA pH 8.0 and fresh ligand was immobilized as described above (one immobilization/regeneration cycle for each analyte concentration). All binding experiments were performed on a Reichert SR 7500DC biosensor at 20°C and a flow rate of 40 μ l/min. Equilibrium binding analysis was performed using Graph Pad Prism 6.0.

Peptidase activity assay by liquid chromatography-tandem mass spectrometry (LC/MS/MS)

The assay was essentially performed as described in (Justa-Schuch et al. 2016). 50 μ M of the BRCA2₁₋₄₀ peptide was incubated alone or in the presence of 125 nM DPP9. To test for inhibition, 10 μ M peptide inhibitor (SLRFLYEG) was added. Reactions were stopped after 6

hours by dilution and acidification in aqueous 0.1% formic acid, 2% acetonitrile (1/20.000, v:v). In addition, bovine insulin was added to the dilution buffer at a concentration of 1 pmol/ul to prevent analyte loss due to adsorption. The resulting samples were analysed on a nanoLC425 nanoflow chromatography system coupled to a TripleToF 5600+ Plus mass spectrometer of QqToF geometry (both AB SCIEX). 5 µl of sample were pre-concentrated on a self-packed Reversed Phase-C18 precolumn (Reprosil C18-AQ, Pore Size 120 Å, Particle Size 5 µm, 4 cm length, 0.15 cm I.D., Dr. Maisch) and separated on a self-packed Reversed Phase-C18 microcolumn (Reprosil C18-AQ, 120 Å, 3 µm, 15 cm, 0.075 cm) using a 15 minutes linear gradient (5 to 50% acetonitrile, 0.1% formic acid modifier, flow rate 300 nl/min, column temperature 50°C) followed by a 5 minutes high organic cleaning step and a 15 minutes column re-equilibration. The eluent was introduced to the mass spectrometer using a Nanospray III ion source with Desolvation Chamber Interface (AB SCIEX) via a commercial Fused Silica tip (FS360-20-10-N-C15, New Objective) at a spray voltage of 2.2 kV, a sheath gas setting of 15 and an interface heater temperature of 150°C. The MS acquisition cycle consisted of a 500 ms TOF MS survey scan that was used for profiling of substrate and product concentrations followed by data-dependent triggering of up to 5 100 ms TOF product ion spectra to confirm the identity of detected analytes. Data analysis was performed using the Analyst TF 1.7 and the PeakView 2.1 softwares (AB SCIEX). Analyses were performed in quadruplicate.

Protein crystallography

Purification, crystallization and structure solution of DPP8 and DPP9 was performed as published in our previous report (Ross et al. 2018). Briefly, the DPP8 isoform 1 (Uni-ProtKB Q6V1X1) and DPP9 isoform 2 (Uni-ProtKB Q86TI2-2) were expressed in *Spodoptera frugiperda* cells and purified. The crystallization was performed in both cases using the hanging drop method with 0.46 M Na-citrate pH 6.75 as precipitant solution and 10 mg/ml of

DPP8 at 4 °C. DPP9 crystallization occurred at 20 °C, using 20 mg/ml of protein and 10% PEG 8000, 25% glycerol, 0.16 M Calcium acetate, and 0.08 M cacodylate pH 6.25 as precipitant solution. Crystals of space group C222₁ (DPP8) and P12₁1 (DPP9) were soaked with BRAC2₁₋₄₀ peptide performing dropping using a pico-dropper (Ross et al. 2018). Data were collected at SLS-X10SA and solved to 3.2 Å and 3.0 Å, respectively, by molecular replacement using a DPP8 structure (PDB: 6EEO). Data processing statistics and refinement values are summarized in table 1. PDB: 6QZW shows DPP8 bound to a dipeptide (MP) from the N-terminus of BRCA2. PDB: 6QZV shows DPP9 bound to a dipeptide (MP) from the N-terminus of BRCA2.

Cycloheximide (CHX) chase assays

HeLa WT cells were seeded at 0.5x10⁶ density in 6 well plates and grown for 24 hours. Next, 300 nM MMC was added to the cells following an additional 24 h incubation. The CHX chase was started by the addition of 100 µg/ml CHX. Where stated, 100 µM of MG132 was added to the cells 30 min before addition of the CHX. Samples were harvested at the indicated time points directly in Laemmli Sample Buffer. Before loading on SDS PAGE, samples were incubated for 10 min at 65°C followed by 4 pulses of sonication at 1% intensity to allow better detection of BRCA2. Immunoblotting and incubation with primary antibodies were performed according to standard protocols. Secondary fluorophore-coupled antibodies were applied. Signals were developed in the Odyssey Sa infrared Imaging System (LI-COR) and analysed with the ImageStudio (version 4.0.21, LI-COR) software.

Chromatin fractionation

HeLa WT and HeLa DPP9 KD were seeded in 6 well plates. The next day (90% confluence), cells were treated with MMC (300nM) for 0, 8 or 24 hours. Chromatin fractionation was

performed essentially as described in (Kari et al. 2016). Briefly cells were re-suspended in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, and protease inhibitors) and centrifuged at 1500 g for 5 minutes. The nuclear pellet was lysed in nuclear lysis buffer (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors) for 30 minutes on ice. Soluble chromatin fractions were separated by centrifuging at 1700 g or 5 minutes. Chromatin fractions were sonicated in water sonicator (BioRad) for 15 min before loading on SDS–PAGE electrophoresis. Immunoblotting and antibody incubations were performed as described above.

Immunofluorescence

HeLa and MEF cells were grown on coverslips in 24 well plates, fixed with 4% formaldehyde in PBS and permeabilized with 0.2% Triton-X-100 in PBS for 5 minutes. Cells were washed with PBS and blocked with 2% BSA in PBS for 10 minutes. Both cell lines were incubated with primary antibodies for 90 minutes at 37°C. Following a PBS wash, cells were incubated for 45 minutes at room temperature with the respective secondary antibodies. Cells were washed with PBS and water and mounted in fluorescent mounting medium (DAKO) with DAPI. Control samples were treated with secondary antibodies only, to estimate background staining. Cells were analysed and images were taken using either an LSM 510-Meta confocal microscope, oil immersion objective 63x/1.3 (Carl Zeiss MicroImaging, Inc), or a Nikon Eclipse Ti2-E Inverted microscope, Plan Apoλ oil immersion objective 60x NA1.40 WD=0.13 (Nikon Instruments Inc). Images were processed using LSM image Browser (Carl Zeiss MicroImaging, Inc) or NIS-Elements AR 5.02.00 (Nikon Instruments Inc), based on the microscope used, and FIJI for the preparation of figures. RAD51 foci were quantified using the Duolink ImageTool (SIGMA-Aldrich).

Proximity Ligation Assay

Proximity Ligation Assay (PLA) was performed using the DUOLINK In Situ PLA Kit from Sigma Aldrich, according to the manufacturer's protocol. In short, HeLa cells were grown on coverslips and fixed as described above for the Immunofluorescence assays. In the case of DPP9 inhibition studies, 10 μ M 1G244 were added for 30 minutes to the cells before fixation. Control cells were mock treated with DMSO. HeLa cells were then incubated with primary antibodies, 90 minutes at 37°C and actin filaments were simultaneously counterstained with CytoPainter Phalloidin-iFluor 488 Reagent (Abcam - #ab176753). Control slides were treated with only one primary antibody to estimate background staining in each experiment. Next, coverslips were washed with PBS and treated with the PLA reagents. Cells were mounted in DAKO with DAPI fluorescent mounting medium and analysed using a LSM 510-Meta confocal microscope, oil immersion objective 63x/1.3 (Carl Zeiss MicroImaging, Inc) or a Nikon Eclipse Ti2-E Inverted microscope, Plan Apo λ oil immersion objective 60x NA1.40 WD=0.13 (Nikon Instruments Inc). Images were processed using LSM image Browser (Carl Zeiss MicroImaging, Inc) or NIS-Elements AR 5.02.00 (Nikon Instruments Inc), based on the microscope used and subsequently analysed using the Duolink ImageTool (SIGMA).

Viability assay

Cells were seeded in 96-well White Microplates (Perkin Elmer cat# 6005680) and treated with different concentrations of Olaparib for 96 hours. For the MMC assays, cells were first incubated for 18-24 h to allow cell attachment. Next, different MMC concentrations were added and cells were analysed 72 hours later. Control cells were treated with DMSO. Cell viability was measured using the CellTiter- Glo® Luminescence Cell Viability Assay (Promega, cat# Cat G7571). CellTiter-Glo® Reagent was added in a 1:1 ratio to the cell culture medium in a well. The plate was placed on an orbital shaker for 10 minutes for induction of

cell lysis. Subsequently, the luciferase signal was measured on a LuminometerDLReadyTMCentro LB 960 reader. Each experiment was performed three times, in triplicate.

Colony Formation Assay

To calculate the respective surviving fractions (SF) after γ radiation (0, 1, 2, 4, 6 and 8 G), a standard colony-forming assay was performed, as previously described (Rave-Fränk et al. 2007). Briefly, cells were exposed to γ - radiation and incubated for 7 days. Next, cells were fixed with 70 % ethanol and stained with Mayer's hemalum (Cat#1.09249.0500, Merck). Non-irradiated cultures were used for normalization. Colonies with >50 cells were scored as survivors. The experiments were performed three independent times, each time in triplicate, and calculated as the median. To validate statistical differences between control and treatment groups, analysis of variance (ANOVA: Two-Factor with Replication) was performed using Microsoft Excel software (version 2016 MSO). P-values < 0.05 were scored as significant.

Accumulation and recovery of γ H2AX signals

Cells were seeded on 96-well, black/clear, tissue culture treated plates (Corning Falcon cat# 353219). To allow cell attachment, cells were incubated at 37°C 5% CO₂ for 18 hours. Next, cells were treated with 250 ng/ml NCS for 30 minutes and allowed to recover in fresh medium for the time points indicated. Cells were then fixed in 4% formaldehyde in PBS for 20 minutes and permeabilized with 0.2% Triton-X-100 in PBS for 20 minutes. Cells were washed with PBS and blocked with 10% FBS in PBS for 30 minutes. The plates were incubated with primary antibodies for 2 hours. Following three PBS wash steps; cells were incubated for 60 minutes with the respective secondary antibodies and 1:5000 DAPI. The γ H2AX signal intensities were measured on a Celigo 4 Channel Imaging Cytometer (Nexlecom Bioscience). The image was

quantified using the Celigo Software. Images were taken using a Nikon Eclipse Ti2-E Inverted microscope, Plan Apoλ oil immersion objective 100x NA1.45 WD=0.13 (Nikon Instruments Inc) and processed using NIS-Elements AR 5.02.00 (Nikon Instruments Inc), and FIJI (ImageJ) for the preparation of figures. These experiments were performed at least 3 independent times, each time with duplicates and calculated as the mean.

Statistical analysis

All graphs were generated using the PRISM8 software, statistical analysis was carried out by unpaired or paired two-tailed t-test, one-way or two-way ANOVA. For protein analysis from immunoblots, ImageStudio software was used after developing of the membranes by LI-COR. Bands were quantified and related to the respective loading control. Mean was calculated by the PRISM software from at least three independent experiments and represented together with SEM error bars. For Colony Formation and Cell Viability assays, measurements from three independent experiments with technical triplicates per data point are represented as mean ± SEM. For microscopy images, dots from PLAs or dots from foci formation assays were quantified with DUO-LINK software.

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