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Formation and nucleolytic processing of Cas9-induced DNA breaks in human cells quantified by droplet digital PCR

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ABSTRACT

Cas9 endonuclease from *S. pyogenes* is widely used to induce controlled double strand breaks (DSB) at desired genomic loci for gene editing. Here, we describe a droplet digital PCR (ddPCR) method to precisely quantify the kinetic of formation and 5′-end nucleolytic processing of Cas9-induced DSB in different human cells lines. Notably, DSB processing is a finely regulated process, which dictates the choice between non-homologous end joining (NHEJ) and homology directed repair (HDR). This step of DSB repair is also a relevant point to be taken into consideration to improve Cas9-mediated technology. Indeed, by this protocol, we show that processing of Cas9-induced DSB is impaired by CTIP or BRCA1 depletion, while it is accelerated after down-regulation of DNA-PKcs and 53BP1, two DSB repair key factors. In conclusion, the method we describe here can be used to study DSB repair mechanisms, with direct utility for molecularly optimising the knock-out/in outcomes in genome manipulation.

1. Introduction

In mammalian cells, DSB lesions are faster repaired through NHEJ, often resulting in small insertions or deletions [1,2]. This has been exploited by the CRISPR-Cas9 technology to generate gene knock-out. Alternatively, in the S and G2 phases of the cell cycle, DSB can be extensively 5′-end nucleolytic processed (a phenomenon called DSB resection) by several endo- and exo-nucleases, in cooperation with helicases, triggering HDR [3]. Notably, from yeast to mammals KU complex and 53BP1 physically antagonize the DSB resection process [3,4]. Concurrently, an intricate network of regulation suppresses DSB resection in G1 cell cycle phase and in post-mitotic cells, thus preventing HDR. Moreover, chromatin context influences the efficiency of induced DSB formation and processing [5]. Once resection starts, DSB cannot be any longer repaired by NHEJ, thus being a critical step to regulate NHEJ and HDR outcomes. Remarkably, misregulation of the balance between NHEJ and HDR triggers genome rearrangements and instability [3]. Indeed mutations in genes encoding factors involved in DSB processing (*e.g.* MRE11, BRCA1, CTIP) have been found in several cancer-prone inherited syndromes. As such, some of those factors are already studied as attractive targets in cancer therapy [6,7]. Importantly, to edit a desired locus in the genome with a donor template (*e.g.* gene sequence substitution or single-codon modifications), resection at Cas9-induced DSB (Cas9-DSB) is compulsory. Therefore, all the regulations and cellular aspects (such as cell cycle phase and chromatin context of the target gene), that influence this crucial step of DSB repair, have to be taken in to account to foresee the success of gene editing protocol. In this scenario, a method to precisely quantify Cas9-DSB formation and processing may be of great utility. In particular, in the literature an open question is how Cas9-DSBs are resected, also considering the relative prolonged binding of the Cas9 protein on the substrate once DNA is cleaved *in vitro* [8,9].

2. Material and methods

2.1. Cell culture, plasmids and treatments

U-2OS and HEK293T cells were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Euroclone). U-2OS-SEC (Stably Expressing Cas9, under the tetracycline-inducible element) and HEK293-SEC (Stably Expressing Cas9, under the tetracycline-inducible element) were available from Professor John Rouse (https://mrcppureagents.dundee.ac.uk, see also [10]), and were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS;

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Gibco), 1% Penicillin/Streptomycin (Euroclone) supplemented with hygromicin B 100 μg/ml and blasticidin 15 μg/ml. U-2OS shSCRAMBLE and sh53BP1 were grown in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Euroclone) supplemented with puromycin 1 μg/ml. In U-2OS-SEC and HEK293-SEC, SpCas9 was induced with 1 μg/ml doxycycline for 24 h before transfection. Plasmid all-in-one vectors px330A-1x2 and px330S-2, carrying *Spcas9* gene and the sgRNAs cloning site, were obtained from Dr. Takashi Yamamoto *via* Addgene (plasmids #58766 and # 58778 respectively [11]. sh53BP1 plasmid was purchased from Sigma-Aldrich (Mission shRNA, NM_005657). siRNA against luciferase (5′-CGUACGCGGAAUACUUCGATT-3′), CTIP (5′-UCCACAACAUAAUCCUAAU-3′) or BRCA1 (5′-CAGCUACCCUUCCAUCAUA-3′) were used for transfection with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. NU7441 compound (a DNA-PKcs inhibitor) was purchased from Selleckchem and was used at a concentration of 5 μM for 24 h after cell transfection with the all-in-one vectors. For resection experiments in Cas9-inducible stable cell lines, sgRNAs targeting DSB1 and DSB2 were synthesized and purchased from ThermoFisher and used for transfection with Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. For experiment with cell nucleofection with the all-in-one vectors, 10^6 U-2OS or HEK293T cells were electroporated with Amaxa Nucleofector II, according to the manufacturer's instructions using the X-001 program and Q-001 respectively.

2.2. Human genomic DNA extraction

U-2OS and HEK293T cells and their derivatives were grown on 6-well plates after transfection. At the indicated time points, cells where trypsinized, washed in PBS and genomic DNA was extracted by NucleoSpin™ Tissue kit (Macherey-Nagel), according to the manufacturer's instructions. The day after, 15 μl of genomic DNA (DNA concentration is around 100 ng/μl) were digested or mock with 20 units of *Bsr*GI or *Bam*HI restriction enzymes (New England BioLabs) for 5 h at 37 °C. Digested or mock DNA was purified and 5 μl were used for the ddPCR reaction.

2.3. Droplet digital PCR assay

The ddPCR reaction was assembled as follows: 5 μl of genomic DNA (approximately 50 ng), 1X ddPCR™ Supermix for Probes (no dUTP, Bio-Rad), 900 nM for each pair of primers, 250 nM for each probe (HEX and FAM, TaqMan probes) and dH_2O to 20 μl per sample. We produced droplets pipetting 20 μl of the PCR reaction mix into single well of a universal DG8™ cartridge for droplets generation (Bio-Rad). 70 μl of droplet generation oil were also added in each well next to the ones containing the samples. Cartridges were covered with DG8™ droplet generator gaskets (Bio-Rad) and then placed into the droplet generator (QX200™, Bio-Rad). After droplet generation, 40 μl of emulsion were transferred from the right well of the cartridge to a 96-well ddPCR plate (BioRad). Before PCR reaction, 96-well PCR plates were sealed with peelable foil heat seals at the PCR plate sealer machine (PX1™, Bio-Rad).

PCR (T100™ thermocycler, BioRad) was run using a ramp rate of 2.5 °C/s between each step. First, Taq polymerase was activated at 95 °C for 5 min and then 39 cycles of 95 °C for 30 s and 58.7 °C for 1 min were made. At the end of the cycles, one additional cycle at 4 °C for 5 min and one at 90 °C for 5 min were made, then temperature was held at 12 °C. After the PCR, FAM and HEX fluorescence was read at the droplet reader (QX200™, BioRad) using QuantaSoft™ software (Bio-Rad). For each sample the number of droplets generated were on an average of 15 000. The number of copies/μl of each target locus was determined setting an empirical baseline threshold identical in all the samples.

For the calculation of Cas9 cleavage efficiency (*CE*), we made the ratio (*r*) between the number of copies of the locus across the Cas9 sites (HEX1 and HEX2 probes) and a control locus on Chr. XXII (FAM probe) in cells transfected with or without sgRNAs. We then calculated:

$$R = r_{+sgRNA}/r_{-sgRNA}$$

and the final *CE* value with the following equation:

$$\%\ Cas9\ cut\ efficiency\ (CE) = (1 - R)*100\%$$

For the measurement of ssDNA generated by the resection process (% ssDNA, *SS* value), we calculated the ratio (*r′*) between the number of copies nearby the DSB loci (335 bp from DSB1 and 364 bp from DSB2, recognized by HEX3 and HEX4 probes respectively) and a control non-target locus (NT locus) on Chr. XXII (FAM probe), with or without sgRNA, digested or mock with *Bsr*GI or *Bam*HI restriction enzymes. The absolute percentage of ssDNA was then calculated with the following equation:

$$\%\ ssDNA\ (SS) = [(r'_{digested}/r'_{mock})_{+sgRNA} - (r'_{digested}/r'_{mock})_{-sgRNA}]\ *100\%$$

The final percentage of DSB resected (*RES* value) was calculated with the formula:

$$RES = SS/CE$$

All the sequences of primers and probes used are listed in Table S1.

2.4. Real Time PCR assay

Real Time PCR analysis was performed as previously described [12]. In brief, 4 μl of digested and undigested DNA was used as template in a 20 μl reaction containing 10 μl 2x TaqMan Master mix (Genespin), 250 nM of each probe and 450 nM of each primer. % ssDNA (*SS*), % Cas9 cut efficiency (*CE*) and % DSB resected (*RES*) were calculated using the formula described in [13].

2.5. Immunoblot analysis

For total protein extract preparation, cells were lysed in 1% SDS sample buffer and protein samples, after loading normalization, were analyzed by SDS-PAGE. Antibodies used in this study were the following: anti-FLAG (clone M2, Sigma-Aldrich), anti-53BP1 (Cell Signaling), anti-GAPDH (Santa Cruz Biotechnology), anti-BRCA1 (Santa Cruz Biotechnology), anti-CTIP (a kind gift from Dr. Pablo Huertas), anti-Actin (Sigma-Aldrich).

3. Results and discussion

DSB resection is a key process to trigger HDR, favouring knock-in outcomes in gene editing. Alternatively, NHEJ pathway promotes limited processing of the break, with addition/removal of one or few bases (indels), often resulting in gene knock-out (Fig. 1A). Aiming to verify and accurately quantify resection at Cas9-DSB, we developed a ddPCR-based protocol. The rational of ddPCR is based on the partitioning of DNA input molecules into thousands of nanoliter-sized uniform droplets that result in an end-point absolute quantification of DNA targets after a standard PCR reaction [14]. Indeed, a ddPCR-based method has been recently described to accurately and precisely measure Cas9-induced cut efficiency in human cell lines [15]. We started from a protocol originally developed in the model organism *S. cerevisiae* to analyse DNA end resection of DSB induced by the homothallic nuclease HO [16,17]. More recently, this was adapted to analyse DSBs induced

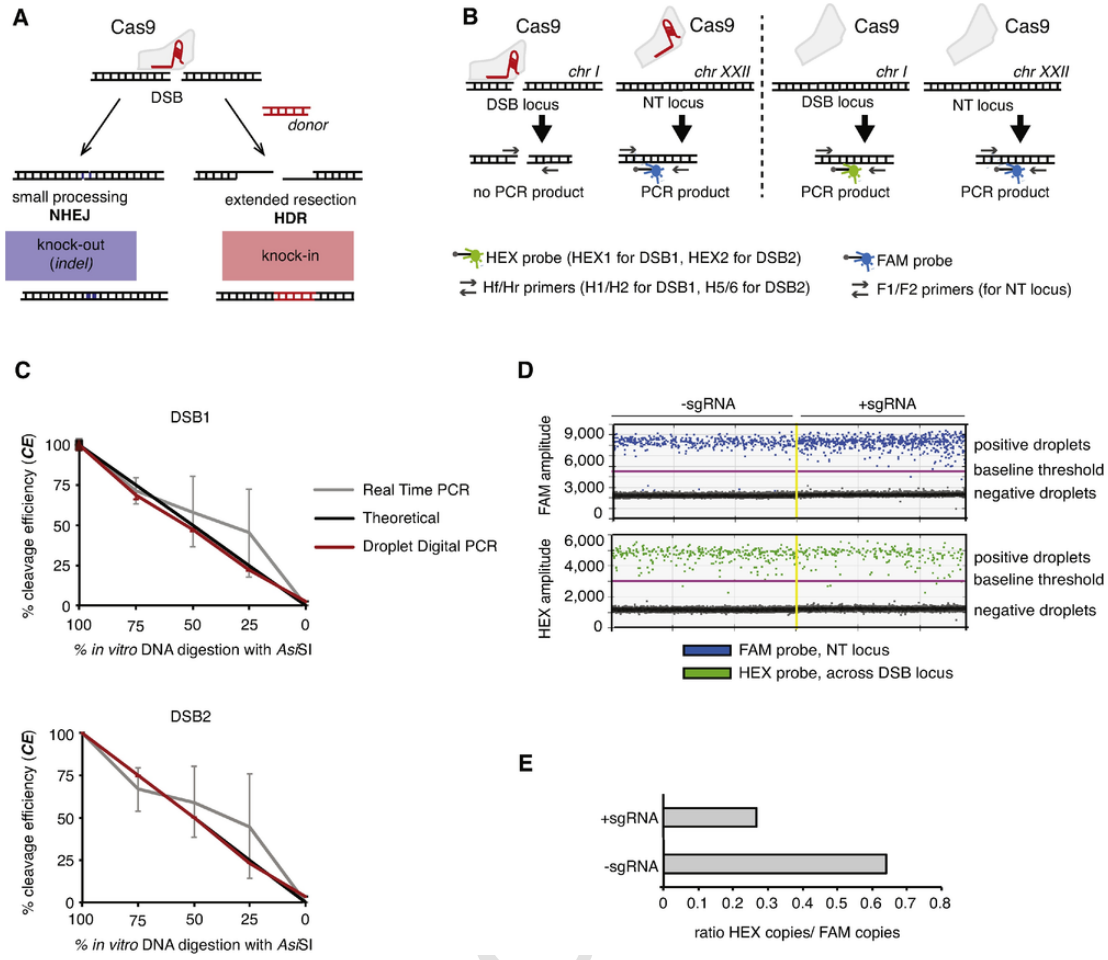

Fig. 1. Cas9-induced DSB formation quantified by a ddPCR-based protocol.A. Schematic representation of the formation of knock-out/in outcomes by Cas9-induced DSB.B. Schematic illustration of our ddPCR assay to calculate Cas9 cut efficiency (*CE*). HEX1 and HEX2 probes are designed downstream or across the selected Cas9-cleavage sites (DSB1, position 89 231 183 on chromosome I; DSB2, position 109 838 221 on chromosome I), whereas a FAM probe is designed in a non-target sequence (NT locus) on chromosome XXII. H1/2 and H5/6 primer pairs amplify a sequence across DSB1 and DSB2, respectively. F1/F2 primer pair amplifies a sequence within the NT locus.C. Genomic DNA was extracted from U-2OS cells and digested or not with *Asi*SI restriction enzyme *in vitro*. Undigested and digested DNA were mixed at fixed proportion (*e.g.* 50% *Asi*SI digested DNA is obtained by mixing equal volumes of undigested and digested DNA) and cleavage efficiency (*CE*) was measured by ddPCR and Real Time PCR (see Material and methods), comparing the obtained values with the theoretical values. In the above plot HEX1 and FAM probes were used, whereas in the lower plot HEX2 and FAM probes were used. The mean ± SD values of two independent experiments are plotted.D. Representative plots for droplets obtained by ddPCR with primer pair H1/H2, 9 h after transfection with/without *in vitro* synthesized sgRNA1 of U-2OS-SEC cells, previously treated with doxycycline for 24 h to induce SpCas9 expression. Droplets are plotted based on their HEX and FAM fluorescence intensity. An empirical baseline threshold is applied to distinguish negative and positive droplets.E. The graph shows the ratio of number of copies of HEX and FAM loci within the same samples described in (D).

by the restriction enzyme *Asi*SI (*Asi*SI-DSB) in U-2OS cells by real-time PCR (RT-PCR) [12,18,19]. Here the protocol has been further modified to quantify the cut efficiency and the 5′-end resection of Cas9-DSB through a novel and versatile ddPCR-based method (ddPCR assay at DSB, or ddPaD). Among the several advantages of the ddPCR over the RT-PCR, there are the high sensitivity and reproducibility of the results, coupled with the use of internal references, without the need of standard curves [20]. Therefore, despite the droplet fluorescence-reading step, overall ddPCR may allow to save time and reagents if compared to RT-PCR (see Material and methods).

In order to set up the method, we chose two *Asi*SI cut sites on Chromosome I, which were already proven to be resected by RT-PCR in U-2OS cell line [12,18,19], to target with specific small guide RNA (sgRNA1 and 2) (Figs. 1B, S1 and S2). To verify if ddPCR would be accurate enough to measure 100% cut efficiency (*CE*) for a completely cut template, we performed an *in vitro* assay digesting or not the genomic DNA with the *Asi*SI enzyme, which cuts in direct vicinity of the target Cas9 sites. Before PCR reactions, digested and undigested samples were mixed at fixed proportion. The empirical *CE* values determined both by RT-PCR and ddPCR were in line with the expected theoretical values (Fig. 1C). Remarkably, ddPCR gave more accurate and reproducible measurements respect to RT-PCR, which is particularly evident when decreasing the amount of amplifiable DNA (50–75% *Asi*SI *in vitro* digested samples, Fig. 1C). This should be taken into consideration when performing *in vivo* analysis, since cleavage induced by heterologous nucleases may be poorly efficient.

The results in Fig. 1C confirm the high reliability of ddPCR compared to RT-PCR, and prompted us to use it to measure Cas9-DSB formation and processing *in vivo*. At first, we transfected a derivative U-2OS cell line (U-2OS-SEC) where SpCas9 gene is stably integrated in the genome and controlled by a tet-OFF promoter [10], with *in vitro* synthesized sgRNA1 and sgRNA2. At different hours after transfection, *CE* was quantified using HEX-labelled probes across the Cas9 cleavage sites (HEX1 and HEX2 for DSB1 and DSB2 loci, respectively) and a FAM-labelled probe (FAM) on a reference non-target locus (NT locus) on chromosome XXII (Fig. 1B). Using ddPCR to amplify the DSB and NT loci, we found that the number of HEX droplets significantly decreased with respect to the FAM droplets once cells were transfected with sgRNA1 and 2. The fraction of positive droplets is then used to calculate the absolute amount of the target sequence (copy) [14], and

the ratio between HEX and FAM copies is reported in a graph (see an example for sgRNA1 at 9 h in Fig. 1D, E). These and other results (data not shown) indicate that Cas9 is cutting the selected DSB1 and DSB2 loci, but not the NT locus. Subsequently, using probes HEX3 and HEX4 we performed ddPCR to amplify a locus 335 bp nearby DSB1 and 364 bp nearby DSB2, after genomic DNA digestion with a selected restriction enzyme (see details in Figs. 2A, S1, S2 and Material and methods). The basic assumption is that once the resection of the 5′-end filament starts, the exposed 3′-end ssDNA filament will be resistant to *in vitro* restriction digestion and therefore will be PCR amplified (Fig. 2A). No-

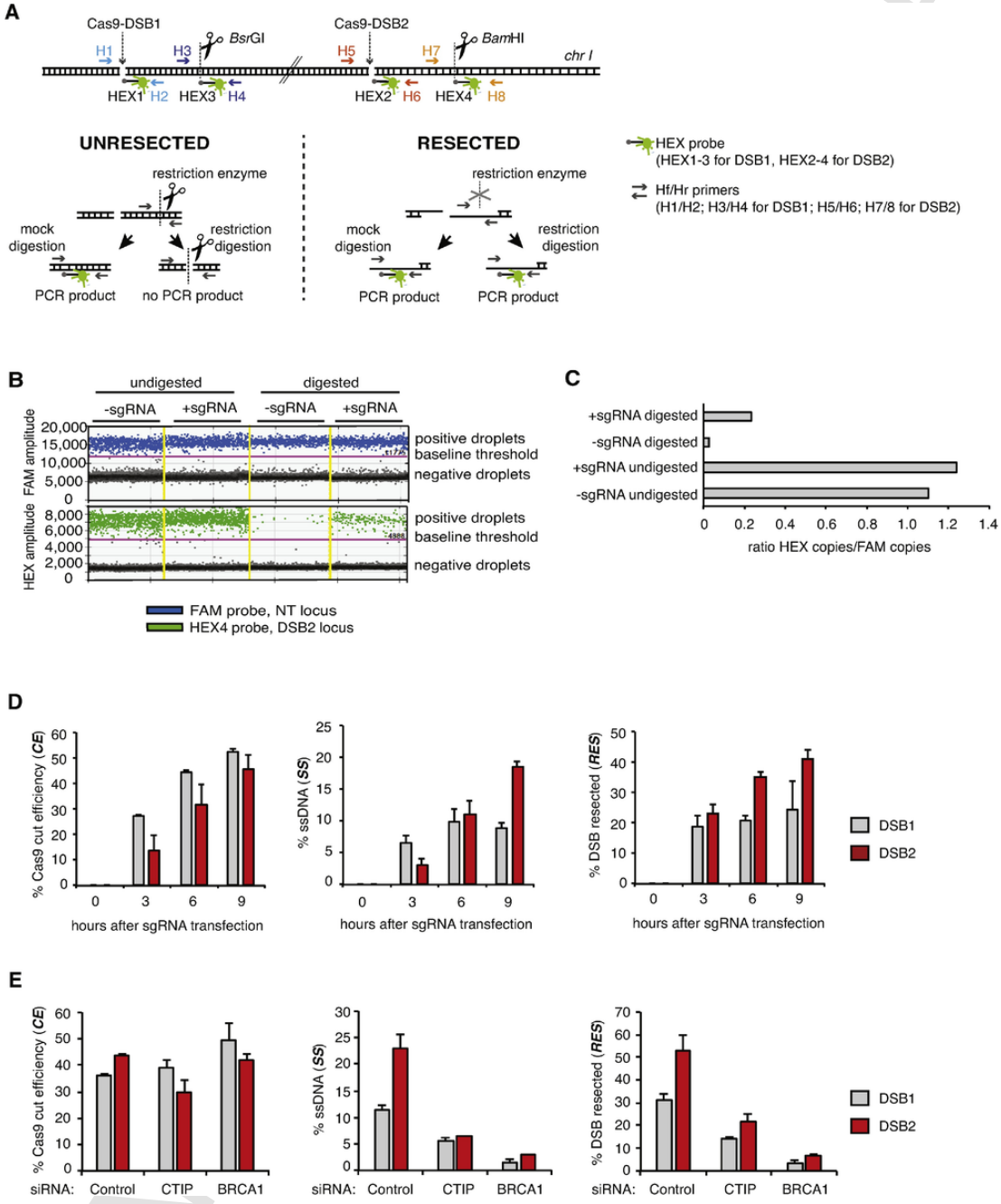

Fig. 2. DSB processing in SpCas9 inducible cell lines quantified by a ddPCR-based protocol.A. Scheme of the strategy used to quantify 5′-end resection nearby a Cas9-DSB. Genomic DNA is extracted and digested with a selected restriction enzyme, which cuts inside the genomic sequence between the primer pair (H3/H4 or H7/H8 for sequences 335 bp nearby DSB1 or 364 bp nearby DSB2, respectively). Synthetic HEX probes (HEX1 and HEX3 for DSB1 and HEX2 and HEX4 for DSB2) are used in the reaction to detect the selected sequence. In particular, after restriction digestion, unresected (double-stranded) DNA sequence will be not amplified.B. Representative plots for droplets obtained by ddPCR with primer pair H7/H8, 9 h after transfection with/without *in vitro* synthesized sgRNA2 of U-2OS-SEC cells, previously treated with doxycycline for 24 h to induce SpCas9 expression. Droplets are plotted based on their HEX and FAM fluorescence intensity. An empirical baseline threshold is applied to distinguish negative and positive droplets.C. The graph shows the ratio of number of copies of HEX and FAM loci within the same samples described in (B). Similar analysis as in (B) and (C) is shown for sgRNA1 in Fig. S3. These values will be used to calculate the percentage of ssDNA (*SS* parameter) at the analysed locus (see Material and methods).D. U-2OS-SEC (Stably Expressing Cas9) cells were treated for 24 h with doxycycline to induce SpCas9 expression. The day after, cells were transfected with *in vitro* synthesized sgRNA1 and 2. Cells were harvested at the indicated time points and genomic DNA was extracted. Undigested or digested DNA samples were analysed by ddPCR using HEX1-4 and FAM probes. The mean ± SD of the *CE*, *SS* and *RES* (% DSB resected) values of two independent experiments are plotted.E. U-2OS-SEC cells were seeded in 6-well plates and the day after CTIP or BRCA1 were silenced by siRNA. After 48 h from siRNA transfection, SpCas9 was induced by doxycycline for 24 h. The day after, cells were transfected with sgRNA1 and 2 as in (D), and after 6 h they were harvested for genomic DNA and protein lisate preparation. The mean ± SD of the *CE*, *SS* and *RES* values of two independent experiments are plotted.

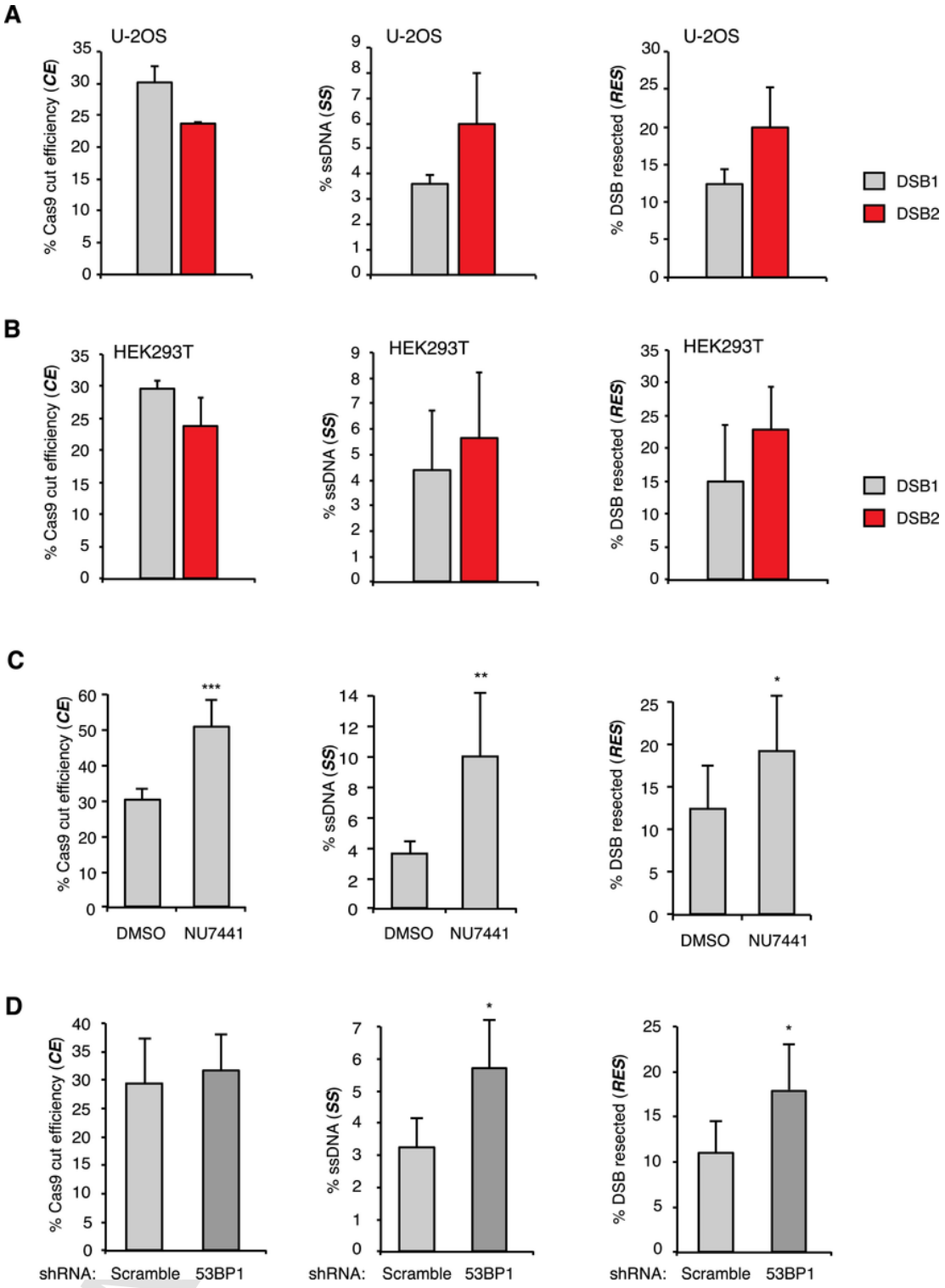

Fig. 3. DNA-PKcs activity and 53BP1 limit Cas9-induced DSB resection in transiently transfected cells.A. Resection of Cas9-induced DSB was analysed at DSB1 and DSB2 in U-2OS cells transfected with all-in-one vectors expressing SpCas9 ± sgRNA1 and sgRNA2. Genomic DNA was extracted 24 h after transfection and ddPCR was performed using appropriate primer pairs, HEX1-4 and FAM probes on undigested or digested samples. The mean ± SD of the *CE*, *SS* and *RES* values of four independent experiments are plotted. The protein level of SpCas9 in the cells is shown in Fig. S3.B. Similar experiment as in (A) performed in HEK293T cell line. The protein level of SpCas9 in the cells is shown in Fig. S3.C. U-2OS cells were transfected as in (A) and cells were treated or mock with NU7441 (5 μM), a commercial DNA-PKcs inhibitor. Genomic DNA was extracted 24 h after transfection and ddPCR was performed using HEX1, 3 and FAM probes on undigested or digested samples. The mean ± SD of the *CE*, *SS* and *RES* values of four independent experiments are plotted. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 according to the T-student test two-tailed.D. U-2OS cells stably expressing scramble shRNA or shRNA against 53BP1 gene were transfected with all-in-one vectors as in (A). Genomic DNA was extracted 18 h after transfection and ddPCR was performed using HEX1, 3 and FAM probes on mock or digested samples. The mean ± SD of the *CE*, *SS* and *RES* values of three independent experiments are plotted. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 according to the T-student test two-tailed. The protein level of 53BP1 in the cells is shown in Fig. S4.

tably, this method cannot distinguish the polarity of nucleolytic processing of the 5′-end filament, which can occur through a bi-directional process [21,22]. As such, at different hours after transfection with the sgRNA1 and 2, and after restriction digestion, the number of HEX3 and HEX4 positive droplets are still quantifiable, while after mock transfection, their signals almost disappear (see examples at 9 h in Figs. 2B and S3A). Importantly, the number of FAM positive droplets at the reference NT locus reflects the amount of DNA in each sample and does not significantly change in all the conditions. This value is then used to calculate the ratio between the HEX and FAM positive copies (Figs. 2B–C and S3A–B). The data indicate that the nucleolytic processing of the Cas9-DSB leads to the exposure of ssDNA at the analyzed loci. We calculate the absolute percentage of ssDNA (*SS* value) at a specific locus as the ratio between the HEX and FAM positive amplicons with or without the sgRNA, after mock sample normalization (see Material and methods). We also calculate the *CE* values at different time points after transfection as described in Material and methods. Then, the ratio between the *SS* and the *CE* values will be hereafter defined as percentage of DSB resected (*RES* value). In Fig. 2D, we plotted the *CE*, *SS* and *RES* values at DSB1 and DSB2 obtained in U-2OS-SEC cell line. We also performed similar analysis in HEK293 derivative cell line [10], stably expressing SpCas9 (Fig. S3E). The same loci nearby the DSB1 and DSB2 have been shown resected after *Asi*SI cutting and analyzed by RT-PCR [12,18], thus validating our results by the new ddPCR approach and clearly showing that the resection of Cas9-DSBs can be precisely quantified, in different cell lines. Remarkably, in a separate experiment in which we directly compared Cas9-DSB formation and processing in U-2OS-SEC cells by ddPCR and RT-PCR, we observed that the two methods gave similar results (Fig. S3F). However, ddPCR was much more accurate and reproducible, according to our previous assay (Fig. 1C).

To further confirm that the ssDNA formation at Cas9-DSB was due to *in vivo* nucleolytic processing, we found that depletion of CTIP and BRCA1 severely reduced *SS* and *RES* values (Fig. 2E), in agreement with other studies [12,19,23]. Of note, using qPCR and different experimental scheme, others did not observe ssDNA decrease in BRCA1 deficient cells (ref 12).

However, the isolation of a single-cell clone stably expressing SpCas9 is not always possible in a reasonable short experimental time frame with all the cell lines of interest, particularly if they are primary cells isolated from patients. Therefore, we applied our ddPCR-based method to quantify formation and processing of Cas9-DSB induced by transient transfection with all-in-one vectors [11], expressing both the SpCas9 and the sgRNAs. We transfected U-2OS and HEK293T cell lines with vectors expressing SpCas9 with or without the sgRNA1 and 2, following a standard electroporation procedure (see Material and methods). 24 h after transfection, we performed ddPCR reactions to determine the *CE*, *SS* and *RES* values, as described above. The results indicate that both the Cas9-DSB1 and 2 can be efficiently induced and resected even after a transient transfection protocol, in the two different cell lines (Fig. 3A,B).

It is well known that the efficiency of knock-in mediated by HDR is substantially lower compared with the efficiency of knock-out mediated by the NHEJ pathway. Suppressing NHEJ or enhancing HDR has been demonstrated to promote the nuclease-mediated knock-in efficiency both in cultured cells and in model organisms [24]. Therefore, we analyzed the efficiency of formation and resection of Cas9-DSB1 in U-2OS cells after treatment with NU7441 (a commercial DNA-PKcs inhibitor), or after silencing the repair and regulatory factor 53BP1 [4]. 24 h after transfection, both the inhibition of DNA-PKcs and the silencing of 53BP1 enhance the *SS* and *RES* values (Fig. 3C,D). Importantly, only the inhibition of DNA-PKcs increases the *CE* values, as a consequence of NHEJ inhibition (Fig. 3C), confirming previous results obtained with similar approaches [12,18].

Overall, the obtained results (particularly the results in Figs. 2E, 3C and 3D) underline the high sensitivity of ddPaD method to quantify Cas9-DSB formation and processing, suggesting a direct utility to further characterize a critical step of the balance between NHEJ and HDR outcome in gene editing, virtually in all cell types and organisms. More in general, ddPaD can be applied to study DSB induced by all type of nucleases that cut in defined loci in the genome. An interesting issue to address in the near future regards the direct correlation between DSB resection and HDR efficiency. This can be investigated at Cas9-DSBs induced in a reporter cassette [25]. Indeed, the regulation of DSB resection is critical for accurate DSB repair, being an attractive target in several basic and medical studies for the identification of novel therapeutic approaches for cancer and other genome instability syndromes [7]. Very relevant for human health, we also propose that the possibility to test the capability to efficiently resect a DSB by a reliable and sensitive method may be utterly informative prior to set up appropriate gene editing protocols to correct pathologic mutations in a desired cell line and, if applicable, in embryos for gene therapy. In fact, mutation in DSB repair factors, cell cycle stage and other cellular features may affect Cas9-DSB processing and editing [24]. Moreover, getting this information for a specific cancer cell type may help to plan personalized therapy to sensitize that specific tumor to pharmacologic-induced DSBs [6]. More in general, we predict that ddPaD method may be useful for all the increasing number of applications, in research and beyond, based on the Cas9 technology [26].

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Ethical standards and informed consent

All the experiments comply with the current laws of the country in which they were performed.

Informed consent was obtained from all individual participants included in this work.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2018.06.005.

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