様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　４ 年　 ６ 月 ２３ 日現在

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研究種目：基盤研究(B)（一般）
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研究課題名（和文）Genome organization as a source of chromosomal instability in cancer.

研究課題名（英文）Genome organization as a source of chromosomal instability in cancer.

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交付決定額（研究期間全体）：（直接経費）　　13,300,000円

研究成果の概要（和文）：DNAは核の中にループ状に折りたまれている。このDNAの圧縮はねじれを引き起こし、トポイソメラーゼII型酵素（TOP2）によって解消される。私は、TOP2がDNAループ形成中にその圧縮を促進していることを発見した。TOP2は通常、DNAのねじれを解消するため一時的に切断し再結合するが、時に切断の再結合に失敗し、DNA損傷や染色体異常、がんをもたらす事が知られている。私は、TOP2の切断がどのように染色体転座につながるかを定量的に調べ、TOP2活性による最初の切断はDNAの折り畳みに依存するが、転写活性がこの切断点処理を制御しゲノムの不安定性や発癌性の染色体転座につながることを発見した。

研究成果の学術的意義や社会的意義
Clarifying the role of TOP2 in genome organization is necessary to understand how genome structure relates to nuclear function. In addition, finding that transcription drives conversion of TOP2 breaks into chromosomal translocations can be used to avoid oncogenic chromosomal aberrations and leukemia

研究成果の概要（英文）：The DNA is packed and folded in loops to fit inside the nucleus. This compression in the DNA leads to torsions that are dissipated by the enzyme topoisomerase type II (TOP2). TOP2 acts in the same locations where the DNA folds to form loops. I found that the role of TOP2 in these locations is to facilitate compaction of the DNA during loop formation. During its normal enzymatic activity, TOP2 temporary cuts and reseal the DNA to relax torsions, but sometimes it fails resealing the breaks and becomes trapped in the DNA. These breaks have to be repaired and can lead to DNA damage, chromosomal aberrations and cancer. I quantified how TOP2 breaks leads to chromosomal translocations and found that although the initial breaks caused by TOP2 activity depend on the folding of the DNA, transcription is responsible of processing these breaks increasing genome instability and leading to oncogenic chromosomal translocations.

研究分野：Genome organization

キーワード：TOPOISOMERASEII　DNA 二重鎖切断　染色体転座発かん　ゲノム安定性　発かん

１．研究開始当初の背景 Initial background to the study

The mammalian genome is folded and packed into loops or topological associated domains (TADs). Loops are formed by the structural maintenance of chromosome complexes (SMC) cohesin. Cohesin loads in the chromatin and translocates along until it encounters a pair of CTCF (CCCTC-binding factor) that stops it. The base of the loop, bound by cohesin and CTCF, is called loop anchor or boundary. Topoisomerase 2 (TOP2) relieves torsional stress in the DNA (supercoiling and entanglements) by producing a transient double DNA break (DSB) and passing another DNA. During this process, TOP2 covalently binds to the ends of the broken DNA forming a short-lived intermediate called TOP2 cleavage complex (TOP2cc). As part of its normal catalytic cycle, TOP2 religates the ends of the DSBs and dissociates without causing any damage. TOP2 poisons such us etoposide or other chemotherapeutic drugs, and sometime spontaneously, increase the levels of TOP2cc inhibiting the ligation step, trapping TOP2 in the cleavage complex. These permanent DNA-protein adducts requires the removal of TOP2 from the DNA ends and the repair of the DSB. These lesions generate genome instability and the oncogenic translocations responsible of secondary leukemias after chemotherapy, like therapy-induced acute myeloid leukemia (t-AML).

I developed a method called END-seq, which detects breaks in the genome (Canela et al. 2016) and I adapted it to map TOP2 transient breaks as a readout of TOP2 activity (Canela et al. 2017 and 2019). I found that TOP2 breaks localize in loop anchors bound by cohesin and CTCF and TOP2 breaks correlate with the binding of cohesin, but it is still unknown the role of TOP2 and the source of the topological stress that TOP2 releases. In the other hand, among the sites of loop anchors and TOP2 activity were the breakpoints of chromosomal translocations found in patients that developed t-AML, suggesting that continuous TOP2 breakage at loop anchors makes them vulnerable to genome instability, but it is still unknown the mechanisms that promote TOP2 breaks to generate chromosomal translocations.

２．研究の目的 Objectives of the study

1. Determine how TOP2 lesions leads to oncogenic chromosomal translocations.
2. Characterize the role of TOP2 in genome organization and loop formation.

３．研究の方法 Research methods

For both objectives, I used Abelson-immortalized mouse preB cells, as they are easily arrested in G1 with the Abelson kinase inhibitor Imatinib (Gleevec) avoiding the background breaks for END-seq of poisoning TOP2 replication, and the toxicity of topoisomerase inhibition during S-phase and mitosis.

To study how TOP2 lesions leads to oncogenic chromosomal translocations. PreB cells were arrested in G1 with Imatinib for 48 hours and then treated with etoposide to map by END-seq total TOP2 lesions/breaks, by a combination of exonucleases ExoVII and ExoT to ensure all DSB ends are free of protein (TOP2cc), or only protein-free DSBs with ExoT alone. Transcription was assayed by nascent RNA-seq. Chromosomal translocations by HTGTS (Hu et al. 2016) expressing a zinc finger nuclease (ZFN) targeting the T cell receptor locus to generate a "bait" DSB to capture by translocation etoposide generated DSBs. For transcription inhibition or stimulation and proteasome inhibition drug pre-treatment was performed for 2 hours prior to etoposide treatment.

To identify the function of TOP2 in genome organization, I used CRISPR/Cas9 gene editing technologies to generate preB cells lines with the AID degron sequence knocked into the genes. PreB cells were arrested in G1 were treated with auxin to degrade TOP2α and cohesin components and used for ChIP-seq and END-seq.

４．研究成果 Research results

1. Determine how TOP2 lesions leads to oncogenic chromosomal translocations.

(i)Investigate how etoposide-dependent DSBs can lead to chromosomal translocations.

To understand the relationship between TOP2 breaks and translocations, we performed high-throughput genome-wide translocation sequencing (HTGTS), a translocation sequencing approach, which relies on the generation of bait DSBs at a defined location, and the subsequent capture of “prey” DSBs (Fig.1A). To generate a “bait”DSB we expressed a zinc finger nuclease (ZFN) targeting the T cell receptor locus in preB-cells, deficient for Lig4 as classical NHEJ suppresses chromosome rearrangements, at the same time of treatment with etoposide (Fig.1B).

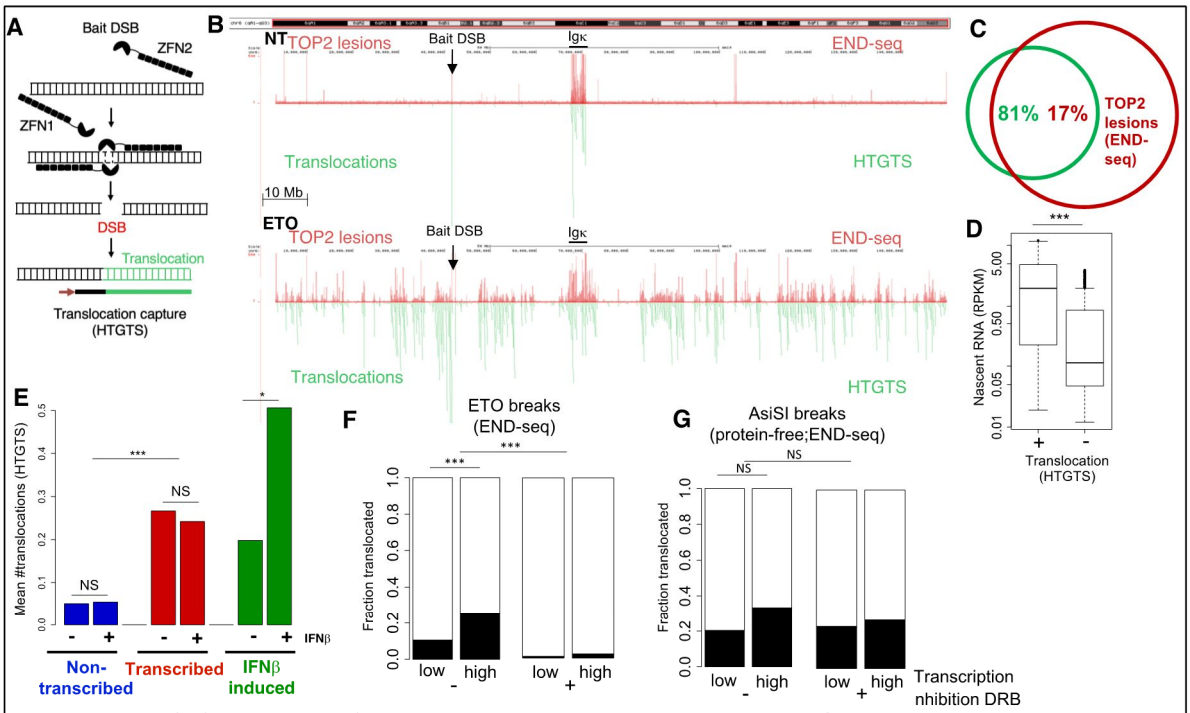

Figure 1. A.HTGTS schema. **B.** Genome profile of chromosome 6 comparing TOP2 lesions by END-seq (red) and translocations by HTGTS (green), in preB cells not treated (NT, upper) and etoposide treated (ETO, lower). Bait DSB is indicated. **C.** Venn diagram showing overlap between translocations in cis to the bait (chr6) (green) and TOP2 lesions detected by END-seq upon etoposide treatment (red). **D.** Nascent RNA-seq levels of TOP2 lesions in chromosome 6 between the lesions that overlap with translocations and the ones that not (*** p<0.0001 t-test). **E.** Barplot comparing mean number of translocations in the presence or absence of IFNB in genes that are not-transcribed, transcribed and INFB induced. **F.** Left: comparison of the fraction of TOP2 lesions that translocate in sites with low transcription (RPKM<0.1) vs high transcription (RPKM>1), right: same but in upon transcription inhibition with DRB. **G.** Same as F but for AsiSI-induced protein-free DSBs.

We found that from all TOP2 breaks in the same chromosome as the bait, only 17% (Fig.1C) exhibited translocations and they were enriched in transcribed areas (Fig 1D). Treatment with interferon beta (IFNB) prior to etoposide resulted in the transcriptional induction of 400 genes and it increased translocations in these genes (Fig.1E). And inhibition of transcription abolished translocations upon etoposide (Fig.1F). Thus, transcription promotes TOP2-mediated translocations. Etoposide induced translocations require removal of TOP2 from the DNA to generate protein-free DSBs, to test how transcription impacts translocation of protein-free DNA ends, we induced translocations expressing the restriction enzyme AsiSI which produces“clean”DSBs. AsiSI-induced DSBs also translocated more at transcriptionally active than inactive broken loci (although not significant), but transcription inhibition did not decrease AsiSI translocations significantly (Fig.1G). Thus, transcription is necessary of translocations of TOP2 breaks compared to those originated from protein-free DNA ends.

(ii) Determine the kinetics of generation and repair of TOP2cc across the genome

To understand how transcription promotes TOP2-mediated translocations, we modified the END-seq assay to discriminate between TOP2-induced DSBs before and after TOP2 has been removed from the break, allowing us to distinguish the proportion of TOP2cc (TOP2 trapped at the DSBs) and protein-free DSBs for every TOP2 break (Fig.2A). The amount of total TOP2 lesions (TOP2cc and protein-free DSBs) in a given site remained constant during the time, but protein-free DSB increased (Fig.2B-C). We noticed that these dynamics were different between genomic locations (Fig.2B), the initial TOP2 lesions (TOP2cc), correlates with cohesin binding, and the conversion rate to protein-free DSB correlates with the level of transcription (Fig.2D). Inhibition of transcription suppressed the enrichment of protein-free DSB in highly transcribed loci (Fig.2E). In conclusion, transcription increases the rate at which trapped TOP2ccs are converted into protein-free DSBs which in turn promotes translocations.

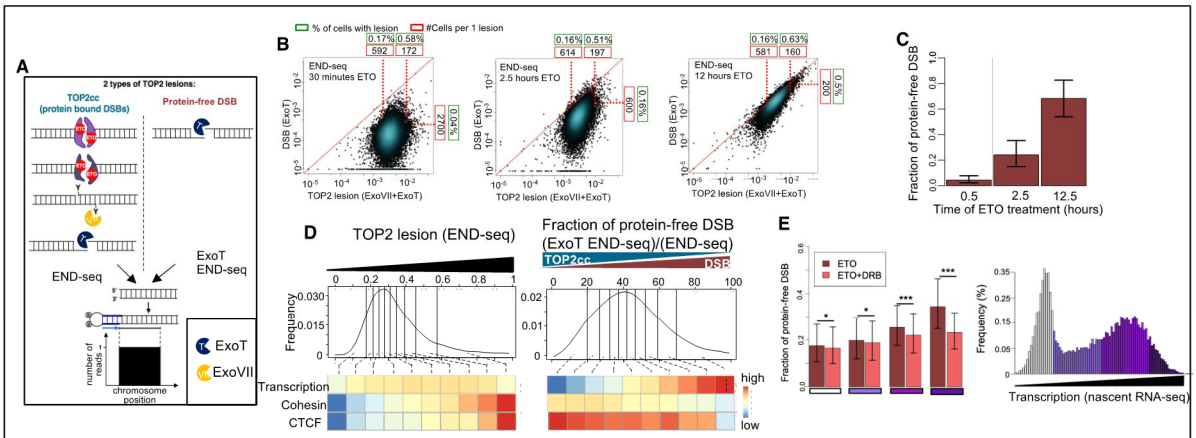

Figure 2. A.Schematic representation of the different TOP2 lesions (left: intact TOP2cc, partially degraded TOP2cc, degraded TOP2cc, right: protein-free DSB) and their detection by END-seq. END-seq uses ExoVII + ExoT to detect all TOP2 lesions, and only ExoT to detect protein-free DSB. **B.** Scatter plots of the levels of END-seq signal using ExoVII + ExoT (TOP2 lesions) and only ExoT (protein-free DSBs) after 30 minutes, 2.5 h and 12 h of ETO treatment. **C.** Ratio of protein-free DSB to total TOP2 lesions for the times of B. **D.** Top: TOP2 lesions divided by deciles by the level of TOP2 trapping (left) measured at 30 minutes post ETO treatment or processing levels (right) measured at 2.5h post ETO treatment. Bottom: average levels for each decile of transcription, cohesin and CTCF binding plotted as a heatmap. **E.** Left: Average fraction of protein-free DSB with and without transcription inhibition (DRB) for each quartile defined in the right. Rigth: Histogram of transcription levels, colors represent each quartile from low and not transcribed (white) to highly transcribed (dark purple)

(iii) Evaluate of the mechanisms of processing and repair of TOP2cc.

Trapped TOP2ccs revert upon washing etoposide from the media. We study how transcription affects to the reversibility by measuring the levels of TOP2ccs before and immediately after etoposide washout, and define the fraction of TOP2cc reversible and irreversible (Fig.3A-B). Most of the TOP2ccs were reversible (88%)(Fig.3A-B). To examine whether transcription affects reversibility we divided the TOP2 breaks into bins according with the level of transcription at their locations in repair deficient cells (Fig.3C-E). We found that the conversion of reversible into irreversible TOP2cc correlates with transcription, but irreversible TOP2cc does not accumulate, they are rapidly converted into protein-free DSB (Fig.3C). The conversion of irreversible TOP2cc to protein-free DBS depends on TDP2, in absence of TDP2 the irreversible TOP2cc fraction accumulates but it is still relative insensitive to transcription (Fig.3D). Thus, transcription mostly affects to the conversion between reversible to irreversible TOP2cc. In addition, we found that proteasome inhibition increased the reversible TOP2cc fraction, and reduced the protein-free DSBs (Fig.3E). Irreversible TOP2cc were also increased may be by post-translation modifications of TOP2cc. In conclusion, transcription accelerates the conversion of reversible TOP2cc to irreversible by proteasome degradation of TOP2.

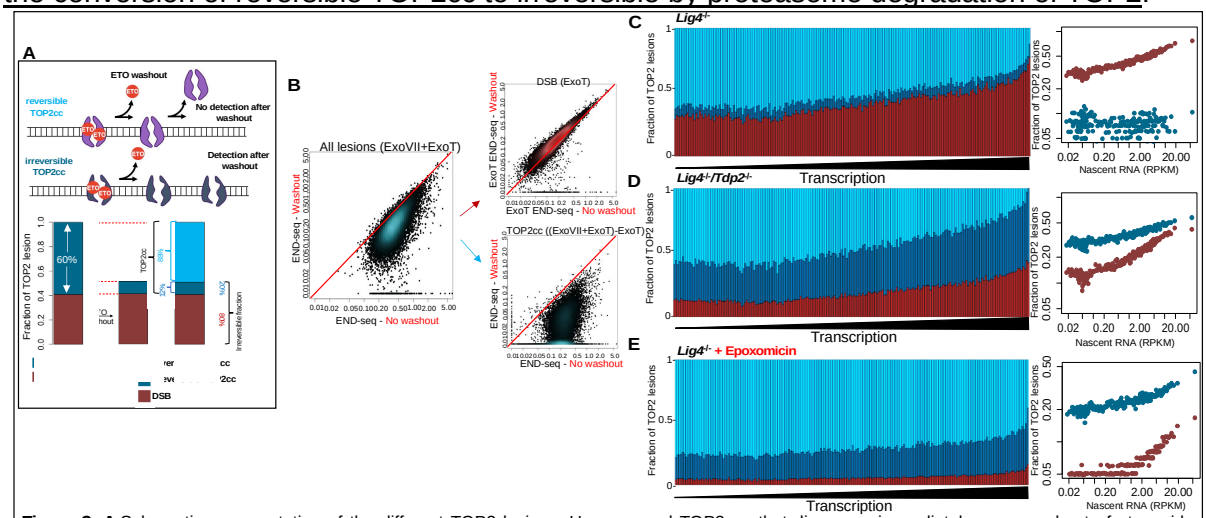

Figure 3. A.Schematic representation of the different TOP2 lesions. Unprocessed TOP2ccs that disappear immediately upon washout of etoposide (ETO) are referred as "reversible TOP2ccs" (light blue). Partially processed or postranslational modified TOP2ccs retained upon ETO washout are referred to as "irreversible TOP2ccs" (dark blue). Fully processed TOP2cc correspond to protein-free DSBs. The fraction of each of these TOP2 lesions shown for Lig4$^{-/-}$ **B.** Scatterplots comparing the levels of TOP2 lesions (ExoVII+ExoT), DSB (ExoT) and TOP2cc (TOP2 lesions - DSB) upon 2.5 hours of ETO before (x axis) and immediately after washout of ETO (y axis). **C-E.** Left: TOP2 lesions in Lig4-/- (C), TDP2-/- Lig4-/- (D) or Lig4-/- cells treated with proteasome inhibitor epoxomicin (E), were divided in 200 bins with increasing levels of transcription, and the relative levels of DSBs (red) and reversible and irreversible TOP2cc (light blue, dark blue and red respectively) were measured for each bin. Right: average levels of transcription plotted against the fraction of DSBs (red) and irreversible TOP2cc (dark blue) per bin for each genotype.

2. Identify the function of TOP2 in genome organization and loop formation.

(i) Analyze genome organization in the absence of TOP2

To analyze the role of TOP2 in cohesin function, I examined cohesin binding by ChIP-seq in absence of any TOP2 activity. Mammals have two TOP2: TOP2α, that is essential for mitosis and expressed only in proliferating cells, and TOP2β, the one acting at loop anchors. I have generated Abelson-immortalized preB cells with an inducible auxin-based degron system (mAID) (Natsume et. al 2016) to deplete TOP2α in cells Top2β-/- arrested in G1 to avoid the toxicity of the lack of TOP2α in dividing cells. I found that absence of TOP2 activity decreases modestly the binding of cohesin by ChIP-seq. Next, I also generated degron preB cell lines for components of cohesin such as RAD21, component of the cohesin ring, WAPL, cohesin unloader, and also for CTCF. First, I combined the degron of WAPL, cohesin unloader, with the absence of TOP2. WAPL depletion accumulates cohesin that aggregates forming axial structures called vermicelli that condensate the chromatin in interphase. I found that upon WAPL depletion the absence of TOP2 decreases cohesin binding and impairs the formation of vermicelli (Fig.4) indicating that TOP2 is necessary for DNA compaction by cohesin. I am now working in understanding why TOP2 activity is needed for vermicelli formation upon WAPL depletion.

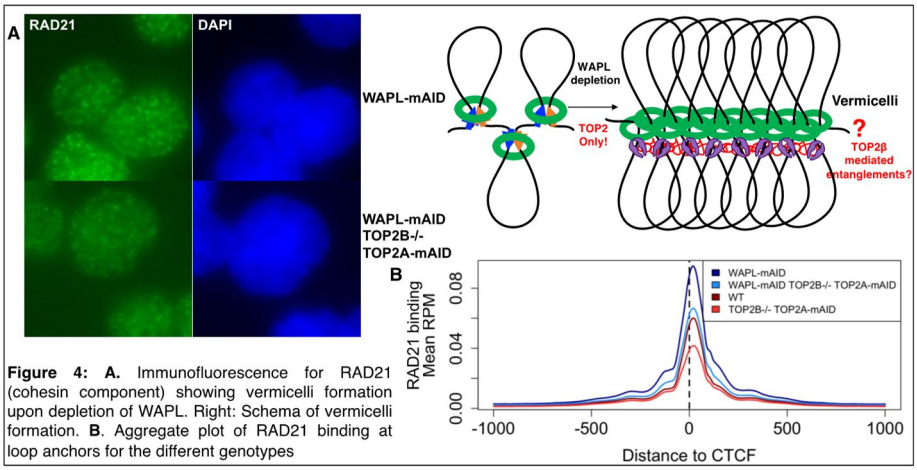

Figure 4: A. Immunofluorescence for RAD21 (cohesin component) showing vermicelli formation upon depletion of WAPL. Right: Schema of vermicelli formation. **B.** Aggregate plot of RAD21 binding at loop anchors for the different genotypes

In parallel, I have generated the degron of RAD21, a component of cohesin, with and without TOP2. And in the same way the degron of CTCF was also generated. Cohesin or CTCF depletion eliminates all chromatin loops but it is rapidly reversible by washing the auxin out from the media, making loops to reappear. I am now evaluating whether these dynamics depends on TOP2 activity.

(ii) Monitor how TOP2 is recruited to loop anchors and how its activity is modulated.

By protein co-immunoprecipitation TOP2β interacts only with the cohesin components SMC1 and SMC3. I could not detect interaction with RAD21, STAG1, STAG2, PDS5 and CTCF. The interaction of TOP2β with SMC1 and SMC3 is not direct, and is mediated by DNA. To evaluate whether TOP2β is actively recruited by cohesin of by the topological stress generated by cohesin constraining the chromatin, I have generated stable preB cell lines with a system to induce the formation of an artificial loop mediated by Cas9, called CLOuD9 (Morgan et al. 2018). This system, called CLOuD9 (Morgan et al. 2018) consists in 2 CRISPR/dcas9 with a dimerization domain tethering two locations in the chromosome. I induced a loop between the beta-globin promoter and the locus control region (LCR) enhancer. I am currently checking the loop formation by 4C (circular chromosome conformation capture) and if TOP2β recruitment by END-seq with etoposide.

(iii) Measure torsional stress across the genome produced by cohesin advancement.

To characterized torsional stress, I have set up:

(1) Negative supercoiling mapping using psoralen-biotin. Psoralen preferentially intercalates in negatively supercoiled DNA and biotin coupling allows to purification and sequencing.

(2) Mapping positive supercoiling by GapR-ChIP-seq. I set up the inducible expression in the preB cells of GapR, a bacterial protein that associates in vivo with positive supercoiled DNA (Guo et al. 2021). The use of a tag allows mapping the binding regions and identification of positive supercoiling by ChIP-seq. As a preliminary data, I detect GapR binding at loop anchors and follows cohesin binding.

５．主な発表論文等

〔雑誌論文〕　計6件（うち査読付論文　6件／うち国際共著　6件／うちオープンアクセス　1件）

１．著者名 Wong N, John S, Nussenzweig A, Canela A.	４．巻 2153
２．論文標題 END-seq: An Unbiased, High-Resolution, and Genome-Wide Approach to Map DNA Double-Strand Breaks and Resection in Human Cells	５．発行年 2021年
３．雑誌名 Methods in Molecular Biology	６．最初と最後の頁 9-31
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/978-1-0716-0644-5_2	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Huang SN, Michaels SA, Mitchell BB, Majdalani N, Vanden Broeck A, Canela A, Tse-Dinh YC, Lamour V, Pommier Y.	４．巻 7
２．論文標題 Exonuclease VII repairs quinolone-induced damage by resolving DNA gyrase cleavage complexes	５．発行年 2021年
３．雑誌名 Science Advances	６．最初と最後の頁 not yet
掲載論文のDOI（デジタルオブジェクト識別子） 10.1126/sciadv.abe0384	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Wu W, Hill SE, Nathan WJ, Paiano J, Callen E, Wang D, Shinoda K, van Wietmarschen N, Colon-Mercado JM, Zong D, De Pace R, Shih HY, Coon S, Parsadanian M, Pavani R, Hanzlikova H, Park S, Jung SK, McHugh PJ, Canela A, Chen C, Casellas R, Caldecott KW, Ward ME, Nussenzweig A.	４．巻 online
２．論文標題 Neuronal enhancers are hotspots for DNA single-strand break repair.	５．発行年 2021年
３．雑誌名 Nature	６．最初と最後の頁 not yet
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41586-021-03468-5	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Canela A, Maman Y, Huang SN, Wutz G, Tang W, Zagnoli-Vieira G, Callen E, Wong N, Day A, Peters JM, Caldecott KW, Pommier Y, Nussenzweig A	４．巻 75
２．論文標題 Topoisomerase II-Induced Chromosome Breakage and Translocation Is Determined by Chromosome Architecture and Transcriptional Activity.	５．発行年 2019年
３．雑誌名 Molecular Cell	６．最初と最後の頁 252-266
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.molcel.2019.04.030	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Shinoda K, Maman Y, Canela A, Schatz DG, Livak F, Nussenzweig A	4．巻 29
2．論文標題 Intra-V? Cluster Recombination Shapes the Ig Kappa Locus Repertoire.	5．発行年 2019年
3．雑誌名 Cell Reports	6．最初と最後の頁 4471-4481
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.celrep.2019.11.088	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

1．著者名 Callen E, Zong D, Wu W, Wong N, Stanlie A, Ishikawa M, Pavani R, Dumitrache LC, Byrum AK, Mendez-Dorantes C, Martinez P, Canela A, Maman Y, Day A, Kruhlak MJ, Blasco MA, Stark JM, Mosammaparast N, McKinnon PJ, Nussenzweig A	4．巻 77
2．論文標題 53BP1 Enforces Distinct Pre- and Post-resection Blocks on Homologous Recombination.	5．発行年 2020年
3．雑誌名 Molecular Cell	6．最初と最後の頁 26-38
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.molcel.2019.09.024	査読の有無 有
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〔学会発表〕　計4件（うち招待講演　3件／うち国際学会　2件）

1．発表者名 Andres Canela
2．発表標題 Topoisomerase II-induced chromosome breakage and translocation is determined by chromosome architecture and transcriptional activity.
3．学会等名 EMBO Workshop: Organization of bacterial and eukaryotic genomes by SMC complexes. Vienna, Austria（招待講演）（国際学会）
4．発表年 2019年

1．発表者名 Andres Canela
2．発表標題 Topoisomerase II-induced chromosome breakage and translocation is determined by chromosome architecture and transcriptional activity.
3．学会等名 EMBO Workshop: DNA topology and topoisomerases in genome dynamics. Les Diablerets, Switzerland（国際学会）
4．発表年 2019年

１．発表者名 Andres Canela
２．発表標題 Genome folding as a source of DNA damage and tumorigenesis
３．学会等名 21st Symposium of Graduate School of Biostudies. Kyoto University（招待講演）
４．発表年 2019年

１．発表者名 Andres Canela
２．発表標題 How to find breaks in the DNA and what they can tell us about genome organization
３．学会等名 Central Dogma Seminar. Graduate School of Biostudies. Kyoto University（招待講演）
４．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関