Multiplexed Detection of Site Specific Recombinase and DNA Topoisomerase Activities at the Single Molecule Level

Felicie Faucon Andersen,[†,§] Magnus Stougaard,[‡,§] Hanne Lærke Jørgensen,[‡] Simon Bendsen,[‡] Sissel Juul,[†] Kristoffer Hald,[‡] Anni Hangaard Andersen,[†] Jørn Koch,[‡] and Birgitta Ruth Knudsen[†,*]

[†]Department of Molecular Biology and Interdisciplinary Nanoscience Center (iNANO), Aarhus University, C.F. Møllers Allé, Building 1130, 8000 Aarhus C, Denmark, and [‡]Department of Pathology and Interdisciplinary Nanoscience Center (iNANO), Aarhus University Hospital, Nørrebrogade 44, Building 18B, 8000 Aarhus C, Denmark. [§]The two first authors contributed equally to the presented work.

ABSTRACT We previously demonstrated the conversion of a single human topoisomerase I mediated DNA cleavage—ligation event happening within nanometer dimensions to a micrometer-sized DNA molecule, readily detectable using standard fluorescence microscopy. This conversion was achieved by topoisomerase I mediated closure of a nicked DNA circle followed by rolling circle amplification leading to an anchored product that was visualized at the single molecule level by hybridization to fluorescently labeled probes (Stougaard *et al. ACS Nano* 2009, *3*, 223—33). An important inherent property of the presented setup is, at least in theory, the easy adaptability to multiplexed enzyme detection simply by using differently labeled probes for the detection of rolling circle products of different circularized substrates. In the present study we demonstrate the specific detection of three different enzyme activities, human topoisomerase I, and Flp and Cre recombinase in nuclear extracts from human cells one at a time or multiplexed using the rolling circle amplification based single-molecule detection system. Besides serving as a proof-of-principle for the feasibility of the presented assay for multiplexed enzyme detection in crude human cell extracts, the simultaneous detection of Flp and Cre activities in a single sample may find immediate practical use since these enzymes are often used in combination to control mammalian gene expression.

KEYWORDS: single-molecule detection · tyrosine recombinases · human topoisomerase I · multiplexing · rolling circle amplification

The demand for highly sensitive and fast high-throughput biological screening systems without the need for complex instrumentation has increased dramatically during recent years and is likely to increase even further in the near future. Especially, the complexity of modern scientific and clinical investigations[1] emphasizes the need for easy-to-handle methods for thorough molecular characterization of often very small crude samples of biological material.

In line with such assay requirements we recently developed a novel single-molecule detection system for the scientifically and clinically important human enzyme, topoisomerase I (TopI).[2] TopI exerts important cellular functions by introducing transient single-stranded DNA breaks in the human genome allowing the relaxation of superhelical tension arising as a consequence of DNA tracking processes.[3] Moreover, the enzyme is of substantial clinical interest, being the sole cellular target for the important anticancer chemotherapeutics, camptothecins, of which the cytotoxic effect has been demonstrated to correlate directly with the intracellular TopI activity level.[4–7] Hence, a robust single-molecule activity assay for this enzyme would be of both scientific and clinical value.

The development of the previously presented single-molecule TopI activity assay was accomplished by TopI-mediated circularization of a synthetic DNA substrate, followed by signal enhancement *via* polymerase-driven rolling circle amplification (RCA). The substrate for TopI comprised a single oligonucleotide, which was converted to a closed DNA circle by a single TopI cleavage—ligation event. For each circularized substrate subsequent RCA resulted in the creation of one rolling circle product (RCP) consisting of multiple (up to 10^3) tandem copies of the circularized TopI substrate.[2,8–10] Each RCP could consequently be optically detected at the single molecule level by hybridization to fluorescently labeled probes followed by microscopic analysis. Using this setup we demonstrated the specific and highly sensitive (10—100 fold enhanced sensitivity compared to standard bulk assays) detection of TopI activity at the single cleavage—ligation event level in purified enzyme fractions or crude extracts from yeast.[2]

Compared to other single-molecule activity assays for TopI, in which magnetic tweezers, optical trapping, or other specialized setups are used to manipulate DNA,[11–15] the RCA-based detection system

*Address correspondence to brk@mb.au.dk.

Received for review September 25, 2009 and accepted November 19, 2009.

Published online December 1, 2009.
10.1021/nn9012912

© 2009 American Chemical Society

ARTICLE

imposes the advantage of being easily adaptable to multiplexed enzyme detection. This can be accomplished simply by using differently labeled probes to detect RCPs generated from different DNA substrates specifically circularized by different enzyme activities. At least theoretically, the only limitation of multiplexed enzyme detection using the RCA-based setup is the available number of different optical nanobeads, and this number is continuously increasing.[16]

In the present study we demonstrate the specific detection of two TopI related enzymes, Flp and Cre recombinase, at the single-molecule level using an RCA-based assay system. Moreover, we present multiplexed detection of either recombinase together with TopI as well as of all three enzyme activities together in crude nuclear extracts from human cells. Besides serving as a proof-of-principle for the feasibility of the RCA-based assay setup for multiplexed enzyme detection in crude biological samples, the specific and multiplexed detection of Flp and Cre activities are likely to find immediate practical use.

Flp and Cre both use the basic TopI cleavage−ligation mechanism[17,18] to mediate highly controllable and conservative recombination between two specific sites (FRT sites for Flp[19] and LoxP sites for Cre[20]) on partner DNA helices, which depending on the composition of the recombined DNA may lead to deletions, insertions, or inversions. These reaction schemes taken together with the fact that Flp and Cre are not naturally expressed in mammalian cells have been successfully utilized to control gene expression in mammalian test systems by inserting the relevant recombination sites and expressing the partner recombinase in a controlled manner.[21,22] Moreover, during recent years a massive effort has been put into the development of Flp- or Cre-based gene integration systems for therapeutic purposes.[23−26]

At present there exist no quick easy-to-handle activity assays with a sensitivity high enough to reliably detect Flp and/or Cre activity in small crude biological samples. Hence, the presented multiplexed single-molecule RCA-based detection of Flp and Cre activities, requiring only the access to a fluorescence microscope, are of anticipated value for both scientific and future clinical applications as a positive control for protocols relying on recombinase activity.

RESULTS AND DISCUSSION

Design of Specific Single-Molecule Detection Assays for TopI, Flp, and Cre Cleavage−Ligation Activities. The experimental setup for single-molecule detection of the cleavage−ligation activities of TopI, Flp, and Cre relies on synthetic DNA substrates (referred to S(TopI), S(Flp), and S(Cre), respectively) designed in such a way that they are converted to single-stranded DNA circles upon reaction with their respective enzyme partners (see Figure 1A).

S(TopI), which was described previously,[2] folds into a double-looped dumbbell-shaped structure of which the double-stranded stem has a preferred interaction sequence for TopI[27] and the properties of a standard TopI cleavage−ligation substrate.[28−30] TopI-mediated cleavage on this substrate occurs three bases upstream to the oligonucleotide 3′-end (indicated by an arrow, Figure 1A) and results in the temporary covalent attachment of the enzyme *via* a 3′-phoshotyrosyl linkage and dissociation of the released three-nucleotide fragment. This allows the 5′-overhang of the substrate to fill in the generated gap, and bring the 5′-hydroxyl group in position for the ligation reaction, which generates a closed DNA circle.

S(Flp) and S(Cre) both fold into single-looped structures with a partly single-stranded stem region having a sequence matching a half FRT- and a half LoxP site, respectively (Figure 1A). Flp and Cre both act in a strictly sequence specific manner with a half FRT site being the minimal cleavage−ligation substrate for Flp[31,32] and a half LoxP site being the minimal cleavage−ligation substrate for Cre.[33] Hence, S(Flp) or S(Cre) is cleaved specifically by Flp or Cre three bases upstream to the 3′-end of the oligonucleotide. For both recombinases, cleavage is accompanied by a temporary covalent attachment of the enzyme to the substrate *via* a 3′-phosphotyrosyl linkage and the diffusion of the short oligonucleotide that is cleaved off. Ligation occurs to the 5′-OH end of the oligonucleotide converting the substrate to a closed single-stranded circle (Figure 1A).

TopI, Flp, or Cre mediated circularization of S(TopI), S(Flp), or S(Cre), respectively, is detected using a solid support assay RCA and fluorescent labeling as described previously.[2,10] The RCA is initiated from a primer (RCA-primer), with a sequence matching the primer annealing sites (denotated "p" in Figure 1B). The RCA-primer is attached to a glass surface to ensure anchoring of the generated RCPs. RCPs are subsequently detected at the single-molecule level by annealing of specific fluorescently labeled probes (detection probes) to an unique identifier sequence of the substrates ("*i*" Figure 1B) and microscopic analysis. It was previously shown that each microscopically detected fluorescent spot represents one RCP, which (since the reaction procedure involves no thermal cycling) in turn represents one closed circle product.[21] Hence, since each cleavage−ligation reaction by TopI, Flp, or Cre generates one closed circle product the presented setup allows the activity of each of these enzymes to be detected at the level of single cleavage−ligation events as previously established for the TopI assay.[2] Moreover, each of the substrates S(TopI), S(Flp), and S(Cre) is designed in such a way that they can be circularized only by their specific target enzyme (TopI, Flp, and Cre, respectively) to allow for multiplexed detection of all three enzymes in a single reaction. This is accomplished in the following manner: For Flp and Cre, which cleave

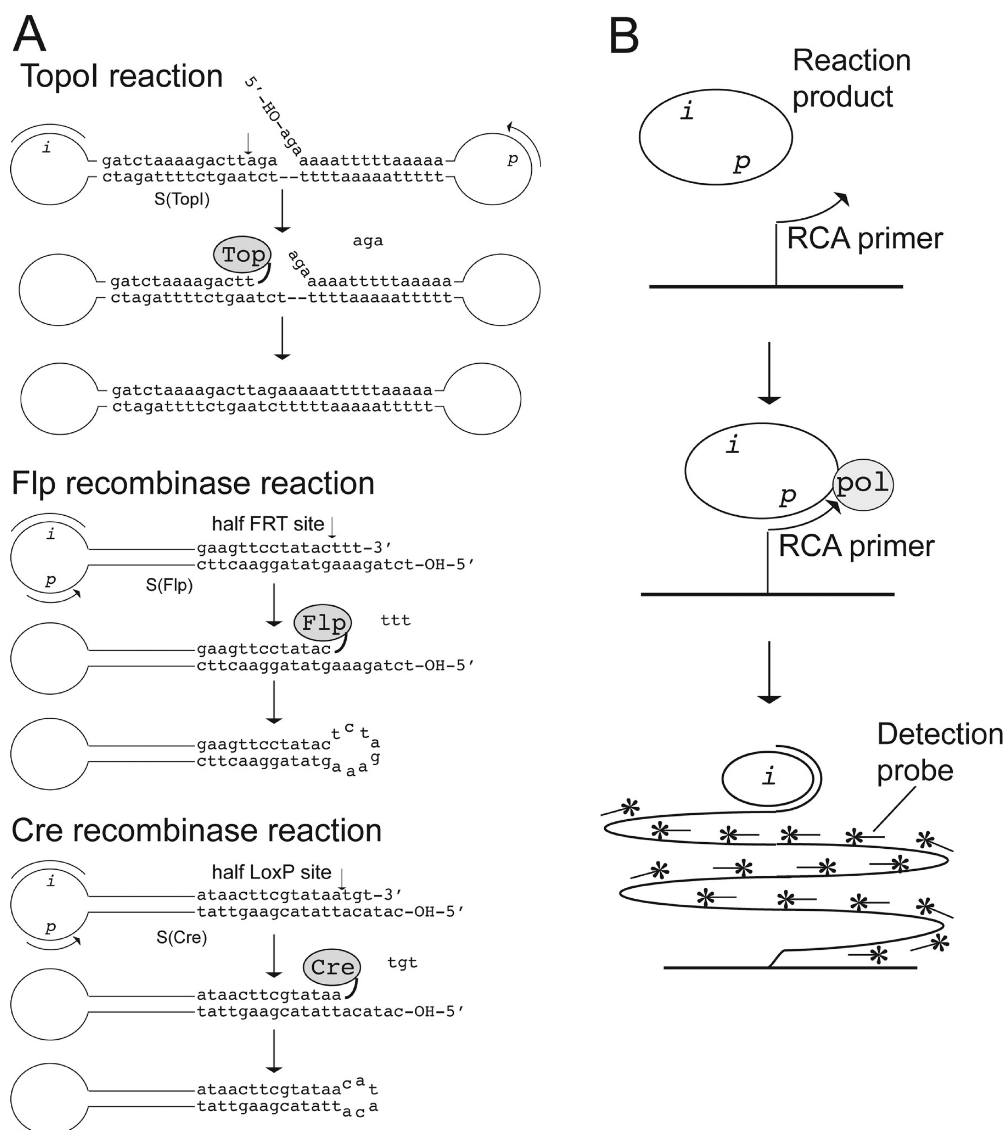

Figure 1. Schematic representation of the solid support RCA-based Flp, Cre, and TopI activity detection assays. (A) The TopI reaction is shown at the top of the figure. S(TopI) folds into a double looped dumbbell-shaped structure, with one loop containing an identifier element (marked *i*), the other loop containing the primer hybridization sequence (marked *p*), and the double-stranded stem region having a preferred TopI recognition sequence (the sequence shown in letter code). The preferred TopI cleavage site is situated three bases upstream to the 3′-end of the dumbbell structure forming oligonucleotide (marked with an arrow) and the 5′-end of the oligonucleotide contains a three-bases overhang matching the noncleaved region of the stem. Note, that the oligonucleotide has a 5′-hydroxyl end making it suitable for TopI-mediated ligation. Following TopI-mediated cleavage at the preferred cleavage site the generated three-nucleotide fragment diffuses away and the 5′-three nucleotide overhang anneals to the noncleavage strand positioning itself for TopI-mediated ligation. TopI seals the nick in the substrate by ligating the 5′-hydroxyl end and dissociates from the substrate. This reaction transforms the open dumbbell-shaped substrate into a closed circle. Since the reaction equilibrium of TopI is shifted toward ligation the formation of the closed circular product is favored. The Flp reaction is shown at the middle of the figure. S(Flp) folds into a partly single stranded loop structure, with the stem forming a half FRT site (shown by letter code), which is the minimal cleavage site for Flp, and the loop containing the identifier element (*i*) and the primer hybridization sequence (*p*). Flp cleavage, which is indicated by an arrow, leads to the formation of a covalent cleavage intermediate and dissociation of the short nucleotide generated during cleavage. Following cleavage the enzyme will ligate the 5′-hydroxyl end of the substrate forming a closed DNA circle. As for TopI the reaction equilibrium is shifted toward ligation favoring the formation of circular products. The Cre reaction is shown at the bottom of the figure. S(Cre) resembles S(Flp) except that the stem part of the substrate contains the sequence (shown by letter code) of half a LoxP site, which is the minimal cleavage substrate for Cre. The reaction of Cre is reminiscent of the Flp reaction and leads to a circular product. Note, that the RCA-primers for all three substrates (indicated by arrows, at the *p* elements) are designed in such a way the polymerase will encounter the strand interruption of the substrate before the identifier element. This is to minimize the risk of false positives in the assay. (B) RCA-based solid support visualization of TopI, Flp, or Cre generated circular DNA products. The *p* region of each of S(TopI), S(Flp), or S(Cre) is hybridized to a 5′-amine linked primer attached to a glass surface, which allows polymerase-assisted RCA of the circularized substrates (on unreacted open substrates the replication reaction is terminated at the strand interruption). Visualization is performed by hybridization of fluorescently labeled oligonucleotide probes to the RCP region corresponding to the *i* element of the dumbbell-substrate. Gray ellipses marked TopI, Flp, Cre, and Pol represents TopI, Flp, Cre, and Phi29 polymerase, respectively. The asterisks represents the fluorophores.

DNA in a strictly sequence specific manner,[17–20] the exclusive reaction of each of these enzymes with their specific substrates is ensured by simple sequence design. TopI, on the other hand, cleaves DNA in a rather sequence independent manner.[3] This enzyme, however, requires a six base pairs stretch of double-stranded DNA spanning positions 6−11 downstream to the cleavage site.[34] This is not a prerequisite for the recombinases.[31–33] Therefore, unspecific cleavage of S(Flp) or S(Cre) by TopI is be prevented by including insufficient length of double-stranded DNA downstream to the cleavage site in these substrates.

Specific Detection of Flp, Cre, and TopI Activities at the Single-Molecule Level Using Purified Enzymes. The feasibility of the described RCA-based detection system as a highly sensitive TopI activity assay allowing specific visualization of single TopI cleavage−ligation events was confirmed earlier.[2] The focus of the present study was to demonstrate specific detection of Flp and Cre activities at the single-molecule level as well as to establish the suitability of the presented experimental setup for multiplexed enzyme detection.

First, the ability of S(Flp), and S(Cre) to serve as specific substrates for their respective enzyme partners was tested using purified recombinant enzymes (Figure 2A). The previously published TopI reaction was included as a positive control in these experiments. The substrates S(Flp), S(Cre), or S(TopI) were incubated with approximately 10 fmol of Flp, Cre, or TopI before RCA. Single-molecule visualization of the resulting RCPs was achieved by the addition of specific fluorescently labeled probes p(Flp), p(Cre), and p(TopI) with sequences matching the "*i*" sequences (see Figure 1) of S(Flp), S(Cre), and S(TopI), respectively. Consistent with FITC-labeling of p(Flp) the Flp reaction resulted in the occurrence of green fluorescent spots (Figure 2B, panel I), while the Cre and TopI reactions, which were visualized by hybridization of RCPs to rhodamine-labeled p(Cre) and p(TopI), respectively, resulted in red fluorescent spots (Figure 2B, panels II−III). Omission of Flp, Cre, or TopI in the reactions resulted in the absence of any signals (data not shown). Moreover, as previously demonstrated for the TopI RCA assay[2] the occurrence of fluorescent signals coincided with the presence of 3′-exonuclease resistant DNA circles in the recombinase reactions (data not shown), strongly supporting that the recombinase activity detection occurs *via* RCA of the circularized substrates as outlined in Figure 1.

To test the specificity of the three substrates, S(Flp) was incubated with TopI and Cre, S(Cre) with TopI and Flp, and S(TopI) with Cre and Flp before RCA and hybridization to the appropriate fluorescently labeled probes. As evident in Figure 2B, panels IV−VI, no fluorescent signals were observed in any of the samples suggesting that, at least between Flp, Cre, and TopI, each of the substrates was circularized to form templates for RCA only by its specific enzyme partner. Finally, the specificity of the probes was tested by probing the sample of panel I with p(Cre) and p(TopI), the sample of panel II with p(Flp) and p(TopI), or the sample of panel III with p(Flp) and p(Cre). The lack of fluorescent signals in samples treated in this manner (panels VII−IX) demonstrated that each probe hybridized to its partner substrate in a highly specific manner.

Note, the aim of the experiments shown in Figure 2 was strictly to demonstrate the specific detection of Flp, Cre, and TopI activities using the RCA-based assay and that quantitative detection of the three enzyme activities was not attempted. Each of the pictures shown in Figure 2 was randomly picked out of a collection of many. The variation in signal numbers between different areas of each microscopic slide (all representing the same sample) approximated the variation in the number of signals between pictures representing different samples (*i.e.*, the variation between panels I−III). No signals were observed in any pictures of the samples represented by panels IV−IX.

Specific Detection of Flp, Cre, and TopI Activities in Nuclear Extracts from Human Cells Using the RCA-Based Activity Assay. For most applications it is imperative that the assay performs well in crude cell extracts since many analyses rely on very small biological samples, which do not allow for enzyme purification.

Cell extracts challenge the assay by containing a diversity of DNA-modifying enzymes, such as ligases and repair enzymes, with a potential capacity for circularizing the utilized DNA substrates and creating false positives in addition to the desired Flp, Cre, or TopI specific signals. Most DNA-modifying enzymes, however, require divalent cations for activity,[35,36] while Flp, Cre, and TopI all perform well in the absence of any cofactors.[3,17,18] Hence, false positive signals caused by unspecific enzyme activities present in cell extracts are likely to be avoided simply by chelating divalent cations by adding surplus EDTA to the reaction mixtures containing crude cell extracts (the ensuing RCA step of the assay requires the addition of Mg^{2+}. However, before this step the circularized products are hybridized to specific primers anchored on glass slides, and cell extracts washed away. Hence, no false positives can occur in this step of the assay). This strategy, indeed, previously allowed the specific detection of recombinant human TopI in crude extracts from yeast cells using the RCA-based detection assay.[2]

Most (if not all) putative scientific or clinical applications of the Flp, Cre, and TopI detection assay described here would require the specific detection of these enzyme activities on a background of mammalian tissue or cell extracts. Therefore, in the present study we addressed the possibility of specifically detecting Flp, Cre, or TopI activities in extracts from human cells. For this purpose we used human embryonic kidney cells (HEK293T cells) either untransfected (for TopI detection)

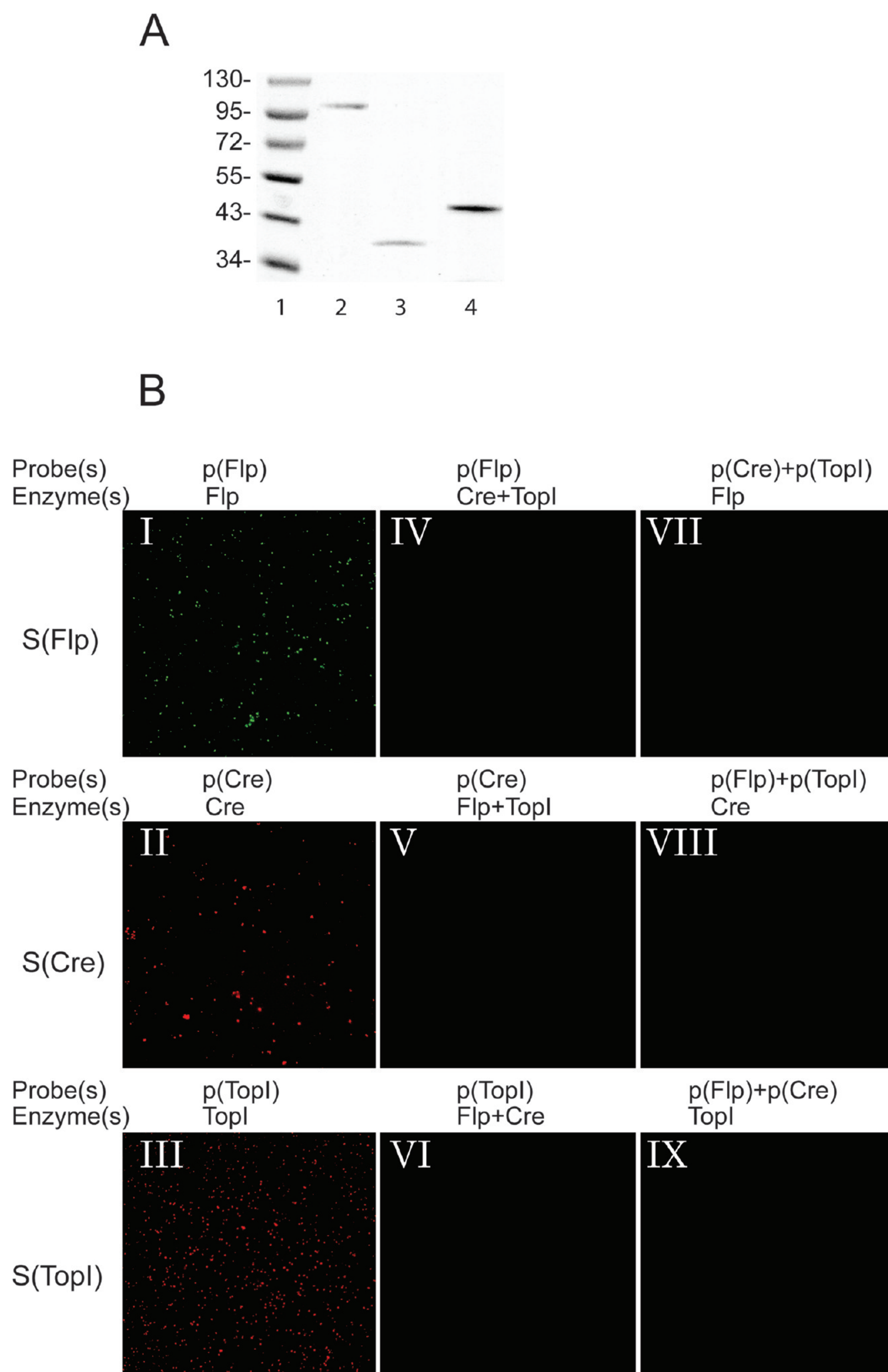

Figure 2. Specific RCA-based detection of Flp, Cre, or TopI using purified enzymes. (A) Coomassie stain of the purified enzymes separated in a 10% SDS polyacrylamide gel. Lane 1, size marker (sizes are indicated to the left of the gel-picture); lane 2, TopI; lane 3, Cre; lane 4, Flp. (B) Solid support RCA-based Flp, Cre, or TopI detection. Panels I−III, the substrates S(Flp), S(Cre), or S(TopI) were incubated with 10 fmol of purified Flp, Cre, or TopI, prior to RCA and microscopic detection of RCPs by hybridization to FITC-labeled p(Flp), or rhodamine-labeled p(Cre) or p(TopI) by fluorescence microscopy. Panel IV−VI are similar to panels I−III except that S(Flp) was incubated with TopI and Cre, S(Cre) was incubated with TopI and Flp, and S(TopI) was incubated Flp and Cre. Panels VII−IX are similar to panels I−III, except that RCPs generated from S(Flp) were hybridized to p(TopI) and p(Cre), RCPs generated from S(Cre) were hybridized to p(Flp) and p(TopI), and RCPs from S(TopI) were annealed to p(Flp) and p(Cre). The utilized substrates are stated to the left of the figure, and the utilized enzymes and probes at the top of the pictures.

or transfected with plasmids allowing the expression of a recombinant thermal stabile mutant of Flp (Flpe)[37] or Cre. Nuclear extracts from these cells were incubated with each of the substrates S(Flp), S(Cre), or S(TopI) in a divalent cation-free buffer. Subsequently, the samples were subjected to RCA, and the resulting RCPs were visualized by hybridization to the appropriate probes (FITC-labeled p(Flp) or rhodamine-labeled p(Cre) or p(TopI)) followed by microscopic analyses.

As demonstrated in Figure 3A, panel I, extracts from cells expressing recombinant Flpe gave rise to green fluorescent spots after incubation with S(Flp), whereas no signals were observed after incubation of this extract with S(Cre) (panel IV). Likewise, incubation of extracts containing recombinant Cre (panels II, V, and VIII), with S(Cre) resulted in the occurrence of red fluorescent signals (panel V) while no signals could be detected upon incubation of this extract with S(Flp) (panel II). Taken together, these results demonstrate the feasibility of detecting both Flp and Cre activity in crude cell extracts using the RCA-based assay and support that no other enzyme activities create false positive signals under the assay conditions. The specificity of the substrates was further confirmed by the lack of signals observed after incubation of extracts from untransfected cells with either S(Flp) or S(Cre) followed by RCA and hybridization to the appropriate fluorescently labeled probes (panels III and VI). Consistent with all extracts containing endogenously expressed TopI, red fluorescent signals were observed upon incubation of either extract with S(TopI) (panels VII, VIII, and IX).

As mentioned, we previously demonstrated the specific detection of recombinant human TopI in crude extracts from yeast *S. cerevisiae* using S(TopI) as a substrate for TopI activity. To address whether the red fluorescent signals observed in Figure 3A (panels VII, VIII, and IX) could be ascribed specifically to TopI activity and were not caused by unspecific enzyme activities present in human cells the experiments were repeated using *in vitro* TopI-depleted human nuclear extracts (Figure 3B).

Endogenously expressed TopI was depleted from nuclear extracts from untransfected HEK293T cells by adding a high molar surplus of a 5′-biotinylated so-called TopI suicide DNA substrate (Figure 3B, left). This substrate acts as a mechanism-based inactivator of TopI by allowing TopI-mediated cleavage, while subsequent ligation is prevented owing to diffusion of the three-nucleotide fragment containing the 5′-hydroxyl end generated during cleavage.[29] Hence, TopI becomes covalently trapped on the suicide substrate and is subsequently removed from the extract by coupling of the biotinylated cleavage complexes to streptavidin-coated magnetic beads. As demonstrated by Western blotting (Figure 3B, middle) this treatment resulted in cell extracts with undetectable levels of free TopI (lane 3), while mock depletion (incubation of cell extracts with only the streptavidin-coated magnetic beads) did not reduce the detectable amount of TopI relative to untreated cell extracts (compare lane 2 with lane 1).

Consistent with the lack of detectable TopI at the protein level, incubation of the TopI-depleted HEK293T nuclear extracts with S(TopI) followed by RCA and hybridization of the resulting RCPs to rhodamine-labeled p(TopI) resulted in only trace numbers of red fluorescent spots (Figure 3B, right, upper picture). Incubation of the substrate with mock depleted extracts, on the other hand, resulted in signals to a level comparable to untreated cell extracts (Figure 3B, right, lower picture. Compare to panels VII, VIII, or IX of Figure 3A). Hence, the number of fluorescent signals detectable in each sample corresponded to the amount of TopI present in the utilized cell extracts strongly supporting the specificity of the TopI RCA-based assay in extracts from human cells.

Note, as with the experiment presented in Figure 2, the results shown in Figure 3 are strictly qualitative and no attempts were made to quantify the activities of Flp, Cre, and TopI. The focus of the performed experiment was to demonstrate the specific detection of each of the three enzyme activities in the background of extracts from human cells.

Multiplexed Detection of Flp, Cre, and TopI at the Single Cleavage−Ligation Event Level. The specific detection of Flp, Cre, and TopI activities in crude cell extracts (demonstrated in Figure 3) coupled with the possibilities of labeling each activity by different color codes offered by the visualization of RCPs by hybridization to specific fluorescently labeled probes holds great promise for multiplexed detection of the three enzymes.

To address the feasibility of multiplexing within the limits of the experimental setup, extracts from cells transfected with the Flpe or Cre expressing plasmids or untransfected cells were incubated with S(Flp) and S(TopI) (Figure 4, panels I−IV) or with S(Cre) and S(TopI) (Figure 4, panels V−VIII) before RCA and hybridization to the appropriate probes. In the experiments containing S(Flp) and S(TopI), RCPs originating from circularized S(Flp) were visualized by hybridization to FITC-labeled p(Flp) while RCPs originating for RCA of circularized S(TopI) were visualized by rhodamine-labeled p(TopI). Hence, Flpe mediated cleavage−ligation events were expected to appear as green fluorescent spots, while TopI mediated cleavage−ligation events would result in red fluorescent spots. As shown in Figure 4, panels I and II, incubation of Flpe containing nuclear extracts with S(Flp) and S(TopI) resulted in the appearance of both green and red fluorescent spots representing Flp and TopI cleavage−ligation of their specific substrates, respectively. This result demonstrates the suitability of the presented RCA-based assay for multiplexed Flp and TopI detection. Moreover, the lack of yellow signals in panels I and II (which would result from a merge of red and green signals) lends

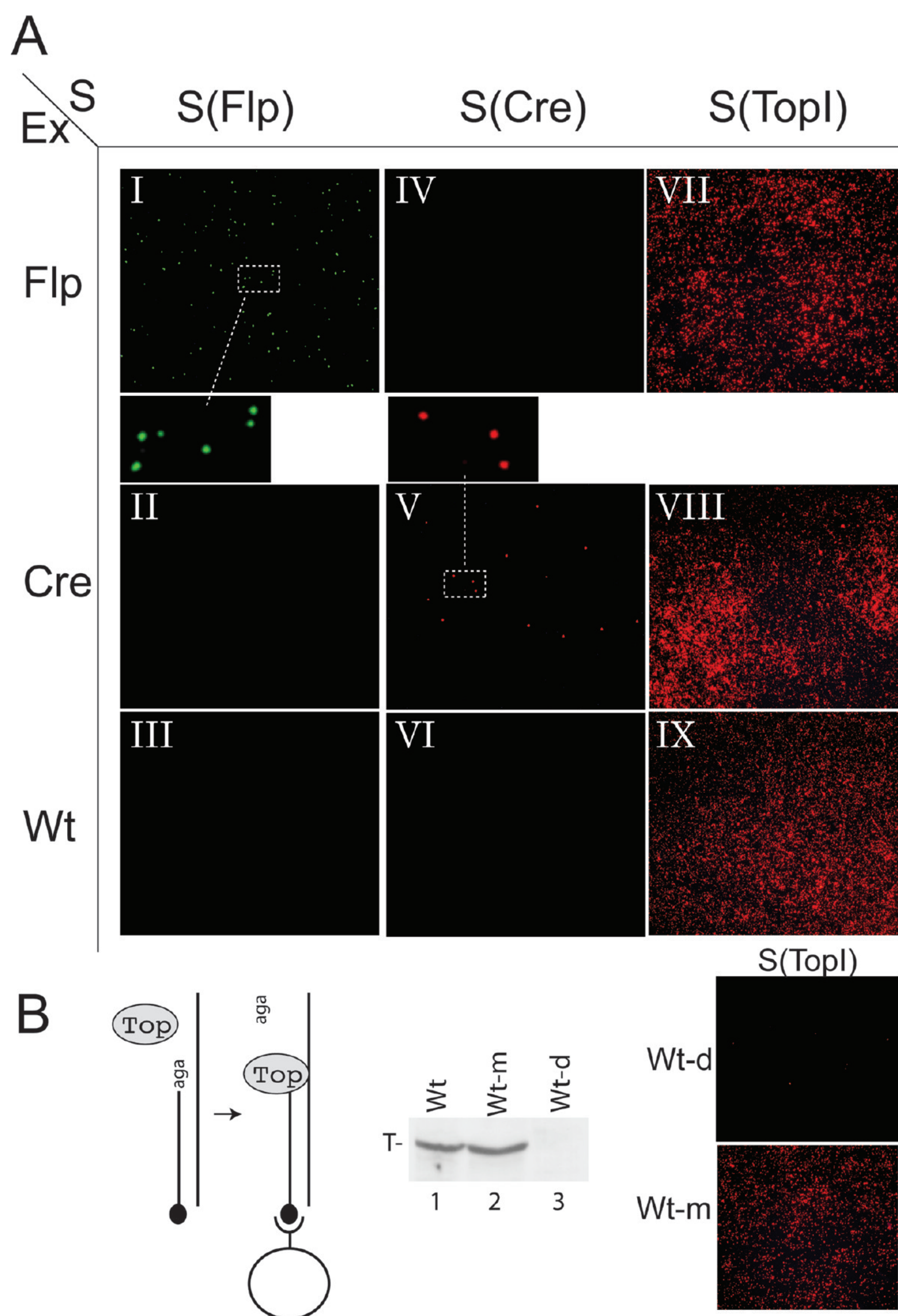

Figure 3. Specific RCA-based detection of Flp, Cre, or TopI in human cell extracts. (A) Solid support RCA-based Flp, Cre, or TopI detection: (Panels I—III) S(Flp) were incubated with nuclear extracts from HEK293T cells transfected with the Flpe expression vector (pCAGGA FLPe), the Cre expression vector (pPGK cre pA), or untransfected prior to RCA and microscopic detection of RCPs by hybridization to FITC-labeled p(Flp). (Panels IV—VI) Same as panels I—III except that S(Cre) was used as a substrate instead of S(Flp) and p(Flp) was replaced by rhodamine-labeled p(Cre). (Panels VII-IX) Same as panels I—III, except that S(TopI) was used as a substrate and RCPs were visualized by rhodamine-labeled p(TopI). (B) Test of the specificity of RCA-based TopI detection in human cell extracts. Left is a schematic representation of the procedure for depleting TopI from the nuclear extracts. A surplus of biotinylated suicide substrate was added to the extract, leading to covalent entrapment of TopI present in the extract to the substrate. Cleavage complexes were removed by precipitation using streptavidin-coupled magnetic beads. Middle depicts nuclear extracts from 2 × 10^6 cells, nontreated (lane 1), mock depleted (lane 2), and TopI depleted (lane 3) analyzed by 10% SDS PAGE, and the TopI in samples visualized by Western blotting using a TopI specific antibody. Right shows RCA-based detection of TopI in the TopI-depleted extract (top panel) and the mock-depleted extract (lower panel). The utilized substrates are stated at the top of the microscopic pictures. The utilized extracts are stated to the left or the right of the pictures. (Flp) extracts from cells transfected with the Flpe expression vector; (Cre) extracts from cells transfected with the Cre expression vector; (Wt) untransfected cells; (Wt-d) TopI depleted nuclear extract; (Wt-m) mock depleted nuclear extract.

further credence to the notion that each spot represents a single RCP, which in turn originates from RCA of a single DNA circle arising from a single cleavage—ligation event. Further supporting the specificity of the

assay only red spots representing TopI cleavage-ligation events were observed upon incubation of S(Flp) and S(TopI) with extracts from cells transfected with the Cre expressing plasmid (panel III) or from untransfected cells (panel IV).

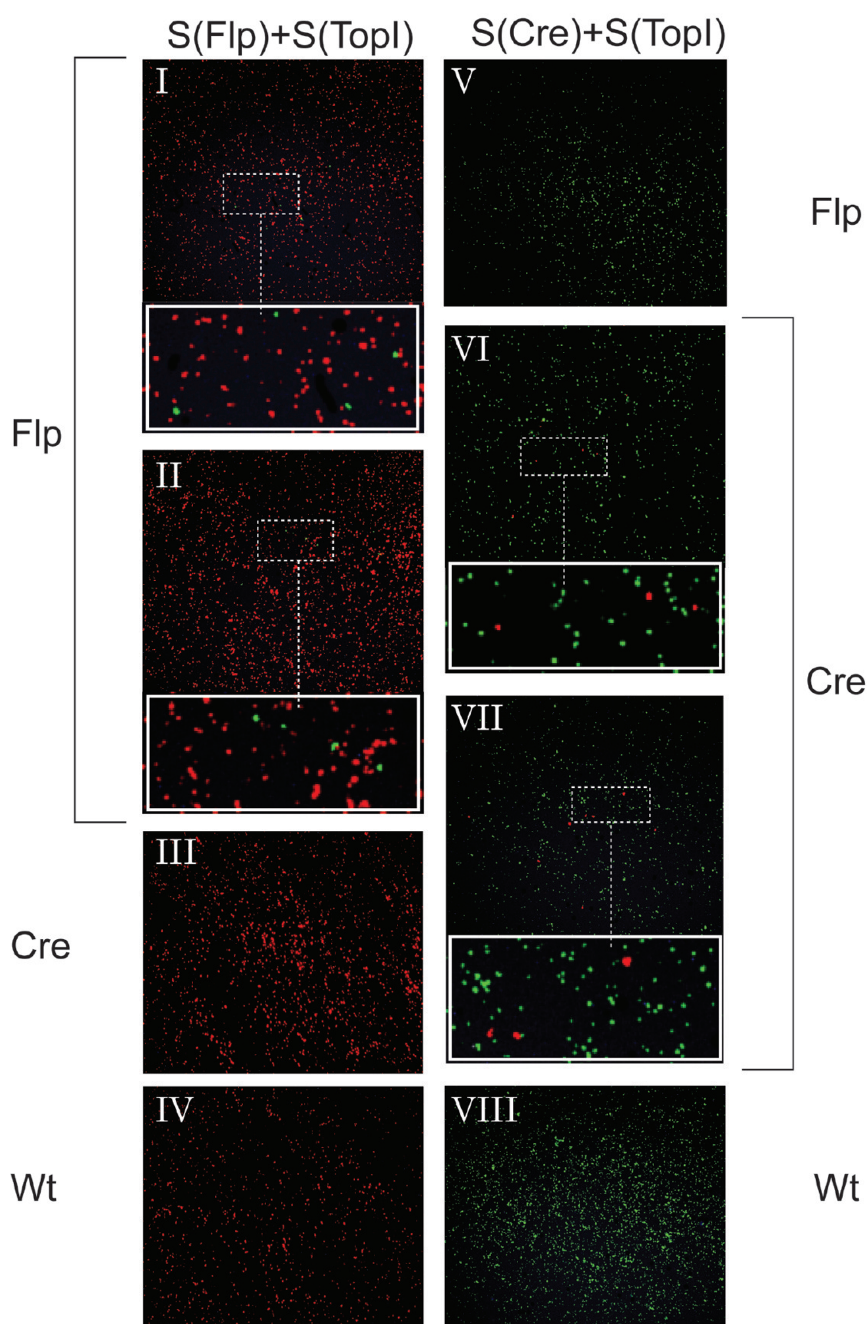

Figure 4. Multiplexed detection of Flp and TopI or Cre and TopI in nuclear extracts from human cells using the RCA-based assay. (Panels I–IV) Microscopic pictures showing the results obtained when a mixture of S(Flp) and S(TopI) was incubated with extracts from cells transfected with the Flpe expression vector (panels I–II), the Cre expression vector (panel III), or untransfected cells (panel IV), prior to RCA and hybridization to FITC-labeled p(Flp) and rhodamine-labeled p(TopI). Blowups of selected areas of the pictures are shown in the lower part of panels I and II for clarity. (Panels V–VIII) same as panels I–IV, except that a mixture of S(Cre) and S(TopI) was used in the assay. RCPs were visualized by hybridization to rhodamine-labeled p(Cre) and FITC-labeled p(TopI). The utilized substrates are stated at the top of the microscopic pictures. The utilized extracts are stated to the left or the right of the pictures. (Flp) extracts from cells transfected with the Flpe expression vector; (Cre) extracts from cells transfected with the Cre expression vector; (Wt) untransfected cells.

In the experiments containing S(Cre) and S(TopI), RCPs originating from circularized S(Cre) were visualized by rhodamine-labeled p(Cre) while RCPs originating from RCA of circularized S(TopI) were visualized by FITC-labeled p(TopI). Hence, in these experiments Cre-mediated cleavage–ligation events would appear as red fluorescent spots, while TopI reactions were expected to result in green fluorescent spots. Consistent with these expectations red fluorescent spots in addition to green spots were only observed upon incubation of the substrate pair S(Cre) and S(TopI) with extracts from human cells expressing Cre (Figure 4, panels VI and VII). This result demonstrates that Cre cleavage–ligation activity, like the Flp activity, can be detected on a background of TopI activity in a multiplexed setup. As for the multiplexed detection of Flp and TopI, the lack of yellow spots in these samples strongly supports that each fluorescent spot represents a single RCP. Moreover, the specificity of the assay was confirmed by the lack of red spots when the S(Cre), S(TopI) substrate pair was incubated with extracts from cells expressing Flpe and endogenous TopI (panel V) or only endogenous TopI (panel VIII). As with the experiments presented in Figures 2 and 3 the pictures shown in Figure 4 were randomly selected out of a collection of many and no direct attempts were made to quantify the activity levels of the three enzymes. However, the simultaneous detection of Flp and TopI or Cre and TopI activities in the multiplexed setup (panels I and II or VI and VII) strongly suggest the activity levels of the recombinases to be much lower than the activity level of TopI in the analyzed cell extracts (Note, the numbers of Flp/Cre specific-signals relative to TopI specific-signals were comparable all over the microscopic slide). Whether this difference is caused by different protein levels in the cell extracts or different activity levels of the enzymes *per se* is not clear. The expression of untagged Flpe and Cre from the commonly used expression vectors[38,39] chosen in this study avoid putative undesired influences of various tags on enzyme

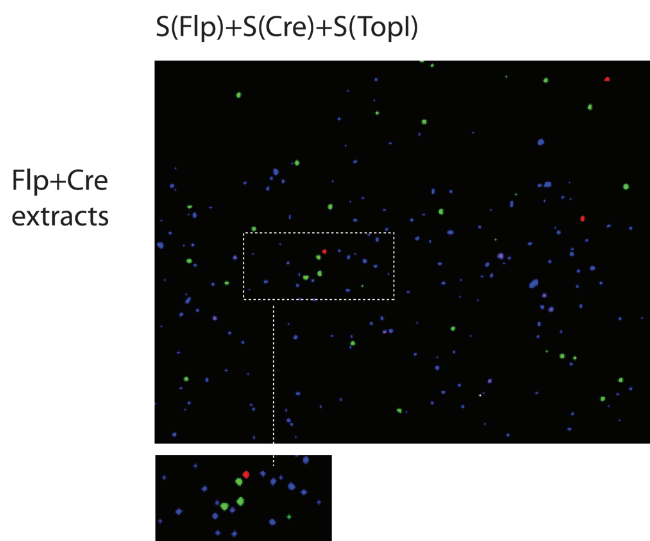

Figure 5. Multiplexed detection of Flp, Cre, and TopI. A mixture of S(Flp), S(Cre), and S(TopI) was incubated with nuclear extracts from HEK293T cells transfected with both the Flpe and the Cre expression vectors prior to RCA and hybridization to FITC-labeled p(Flp), rhodamine-labeled p(Cre), and Cy5-labeled p(TopI).

activity but leave no possibilities of detecting the enzymes at the protein level since antibodies against the native recombinases are not available.

To investigate the possibility of detecting all three enzyme activities in a single sample, extract from cells transfected with both the Flpe and Cre expression plasmids was incubated with a collection of all three substrates S(Flp), S(Cre), and S(TopI) prior to RCA and hybridization to FITC-labeled p(Flp) giving rise to green fluorescence, rhodamine-labeled p(Cre) giving rise to red fluorescence, and Cy5-labeled p(TopI) giving rise to blue fluorescence. As evident from Figure 5, this combination resulted in the occurrence of green, red, and blue spots readily detectable by microscopic analysis and clearly demonstrating the possibilities of detecting all three enzyme activities in one multiplexed analysis. Likewise, multiplexed detection of only Flp and Cre activities, which may be directly applicable for scientific and clinical purposes, was easily achieved in a reminiscent setup simply by omitting S(TopI) from the reaction mixtures (data not shown).

Taken together the presented results demonstrate the feasibility of the substrates S(Flp), S(Cre), and S(TopI), to allow for the specific monitoring of three related enzyme activities, Flp, Cre, and TopI at the single cleavage−ligation event level in purified enzyme fractions as well as in crude extracts from human cells using the RCA-based assay setup. This in turn allowed for the multiplexed detection of two or all three of the enzyme activities in a single analysis simply by using two or three of the specific substrates in combination with detection probes with different color labeling. The multiplexed detection of Flp, Cre, and TopI serves as a proof-of-principle for the simultaneous detection of three different enzyme activities in a single sample, but may be of limited practical use. More applicable may be the simultaneous detection of Flp and Cre activities, since these enzymes are often used in combination to control gene expression in mammalian cells or animal models.[40,41] Hence, the described RCA-based assay may be of immediate practical use for instance in animal-model developmental studies or mammalian cell studies (where Flp and Cre are used to control gene expression and/or gene integration) providing a simple and quick mean of detecting recombinase activity in crude cell samples as a positive control. Also, the solo detection of Flp or Cre in crude biological samples using the simple microscopic visualization technique might be useful for detecting recombinase activity in systems where a single recombinase is used to control gene expression/integration.[23−26,42] The specific detection of TopI activity in human cell extracts may be of substantial cancer prognostic value, as TopI is the sole target for several anticancer therapeutics.[4]

The single-molecule detection of RCPs allowing the visualization of single Flp, Cre, or TopI cleavage−ligation events opens up for the potentially very sensitive measurement of enzyme activity, in principle down to the single cell or even the single enzyme level. A similar sensitivity may be obtained using a PCR-based readout format. However, the thermal cycling principle of PCR would compromise detection of individual cleavage−ligation events and likely the accuracy of the assay, owing to the exponential accumulation of inaccuracies inherent to PCR.

We believe that the presented RCA-based single-molecule detection assay presents unique advantages for both basic and applied science allowing subtle variations between individual enzymes or extracts from individual cells in a large population to be recognized. In line with these considerations we previously demonstrated a 10−100-fold increased sensitivity of the TopI-specific RCA-based detection assay compared to standard bulk TopI activity assays,[2] and we are currently developing means of increasing the sensitivity of our RCA-based enzyme detection even further. However, quantitative enzyme detection, which was presented previously,[2] was out of the scope of the present study where focus was on the development of new RCA-based assays allowing the specific and multiplexed detection of three related enzyme activities. The successful multiplexed enzyme detection presented here, holds promise for the possibilities of developing even more extended setups allowing the simultaneous de-

tection of enzyme collections relevant for different industrial or clinical applications.

CONCLUSION

The RCA-based single-molecule detection system presented here enabled the specific and multiplexed detection of Flp, Cre, and TopI activities present in crude extracts from human cells at the single cleavage−ligation event level. This was achieved by converting each cleavage−ligation reaction mediated by either one of the enzymes on its specific substrate partner to a RCP that could be specifically visualized at the single-molecule level by hybridization to fluorescently labeled probes. To distinguish the activities of the three enzymes differently labeled probes were designed to match the sequences of RCPs resulting from Flp, Cre, and TopI reactions, respectively.

The simultaneous detection of Flp, Cre, and TopI in a single sample serves as a proof-of-principle, showing the possibilities of multiplexed detection of at least three enzyme activities at the single molecule level using the RCA-based setup. The multiplexed detection of Flp and Cre may be of more immediate practical use, since these enzymes are often employed in combination to control gene expression in mammalian cells or animal models. Moreover, besides its potential for basic scientific investigations the presented method holds great promise for clinical applications, as TopI is the primary target for already routinely used anticancer therapeutics, while Flp and Cre are anticipated key-players in future gene-therapeutic protocols.

METHODS

Reagents and Enzymes. Phi29 DNA polymerase was from MBI Fermentas. All oligonucleotides were purchased from DNA Technology A/S, Aarhus, Denmark. CodeLink Activated Slides were from SurModics, and Vectashield was from Vector Laboratories. BL21(DE3) pLysS bacterial strain was from Promega; Streptavidin-coupled magnetic beads were from Dynal.

Yeast Strains and TopI, Flp, Flpe, and Cre Expression Plasmids. The yeast *Saccharomyces cerevisiae* Top1 null strain RS190 was a kind gift from R. Sternglanz (State University of New York, Stony Brook, NY). Plasmid pHT143, for expression of recombinant full-length TopI in yeast was described previously.[28] The plasmid pET11a(Flp) for expression of recombinant Flp in BL21(DE3) pLysS was a kind gift from M. Jayaram (University of Texas at Austin, TX). Plasmids pCAGGA FLPe and pPGK Cre pA for expression of Flpe and Cre in human cells were kindly provided by Francis Stewart (Technische Universitaet Dresden, Dresden, Germany) and Klaus Rajewsky (Division of Pathology, Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA), respectively.

Enzyme and Cell Extract Preparations. The plasmid pHT143 was transformed into the yeast *S. cerevisiae* strain RS190. The protein was expressed, and purified enzyme was prepared as described previously.[28,43] Purification of recombinant Flp was described previously.[44] Purified recombinant Cre was a kind gift from M. Jayaram (University of Texas at Austin, TX). The protein concentrations were estimated from Coomassie blue-stained SDS−polyacrylamide gels by comparison to serial dilutions of BSA.

Human embryonic kidney HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin. Cells were incubated in a humidified incubator (5% CO_2/95% air atmosphere at 37 °C).

Transient transfections of plasmids into HEK293T cells were obtained using lipofectamine2000 (Invitrogen) and 0.5 mM DNA and were carried out in the presence of FBS according to the manufacturer's instructions. Transfection efficiencies as measured by the expression of a green fluorescent protein (GFP) control construct were >80%. Functional assays were conducted 24 h after transfection, and cells were harvested with a cell scraper. Media was discarded and the cell lysed in 1 mL of Lysis buffer (0.1% NP-40, 10 mM Tris, pH 7.9, 10 mM $MgCl_2$, 15 mM NaCl, 0.1 mM phenylmethyl sulfonyl fluoride[45]). After cell lysis the nuclei were extracted in 80 μL of 0.5 M NaCl, 20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM phenylmethyl sulfonyl fluoride.[46] The amount of cells used for extract preparation was estimated to ~4×10^4 cells/mL (extract) by cell counting prior to harvesting.

Synthetic DNA Substrates, Probes, and Primers. Oligonucleotides for construction of the S(TopI), S(Flp), S(Cre), the utilized detection probes (p(TopI), p(Flp), and p(Cre)) and RCA-primers were synthesized by DNA Technology on a model 394 DNA synthesizer from Applied Biosystems. The sequences of the oligonucleotides are as follows: S(TopI), 5′-AGAAAAATTT TTAAAAAAAC TGTGAAGATC GCTTATTTTT TTAAAAATTT TTCTAAGTCT TTTAGATCCC TCAATGCTGC TGCTGTACTA CGATCTAAAA GACTTAGA-AMINE-3′; S(Flp), 5′-TCTAGAAAGT ATAGGAACTT CGAACGACTC AGAATGAGGC TCAATCTAAT GGACCCTCAA TGCACATGTT TGGCTCCCAT TCTGAGTCGT TCGAAGTTCC TATACTTT-3′; S(Cre), 5′-CATACATTAT ACGAAGTTAT GAGCGTCTGA GTATGGCTCA CCAGGACTCT ATGCAGTGAA TGCGAGTCCT TCTACTCATA CTCAGACGCTCATAACTTCGTATAATGT-3′; RCA-primers, matching S(TopI), 5′-AMINE-CCAACCAACC AACCAAATAA GCGATCTTCA CAGT-3′; matching S(Flp), 5′-AMINE-CCAACCAACC AACCAAGTCC ATTAGATTGA GCCT-3′; matching S(Cre), 5′-AMINE-CCAACCAACC AACCAACATA GAGTCCTGGT GAGC-3′; detection probes, p(TopI), 5′-"F"-GTAGTACAGC AGCAGCATTG AGG-3′; p(Flp), 5′-"F"-GGAGCCAAAC ATGTGCATTG AGG-3′; p(Cre), 5′-"F"- AGACGGACTC GCATTCACTG-3′. "F" indicates fluorescent labeling, which was Cy5, rhodamine, or FITC as stated in the text.

For preparation of the biotinylated suicide substrate we used OL19-biotin, 5′-biotin-GCCTGCAGGT CGACTCTAGA GGATCTAAAA GACTTAGA, and OL27-amine, 5′-AMINE-AAAAATTTTT CTAAGTCTTT TAGATCCTCT AGAGTCGACC TGCAGGC. For assembly of the substrate by hybridization, 5 nmol of each of the oligonucleotides were mixed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, heated to 85 °C, and cooled slowly to room temperature. All oligonucleotides used for substrate assembly were gel-purified as described previously.[47,48]

RCA-Based TopI, Flp, or Cre Detection. The 5′-amine-coupled primer was linked to CodeLink Activated Slides according to the manufacturer's description. The TopI, Flp, and Cre reactions were carried out in a 10 μL reaction volume containing a divalent cation depletion buffer (5 mM Tris-HCl, pH 7.5 2 mM EDTA, 8 mM NaCl, 0.6% beta-mercaptoethanol, 10% PEG6000) supplemented with 100 nM of substrate(s) at stated in the text. Reactions were initiated by the addition of 10 fmol of one or more of the purified enzymes TopI, Flp, or Cre or of HEK293T nuclear extracts as stated in the text. Incubation was carried out for 30 min at 37 °C before heat inactivating the enzyme(s) for 5 min at 95 °C. Subsequently, hybridization to the covalently coupled primer was performed for 60 min at room temperature (22−25 °C) Slides were washed for 2 min at room temperature in wash buffer 1 (0.1 M Tris-HCl, 150 mM NaCl, and 0.3% SDS) and for another 2 min at room temperature in wash buffer 2 (0.1 M Tris-HCl, 150 mM NaCl, and 0.05% Tween-20). Finally, the slides were dehydrated in 99.9% ethanol for 1 min and air-dried. Rolling circle DNA synthesis was performed for 45 min at 37 °C in 1× Phi29 buffer supplemented

with 0.2 μg/μL BSA, 250 μM dNTP, and 1ug/μL Phi29 DNA polymerase. The reaction was stopped by washing in wash buffers 1 and 2. The RCPs were detected by hybridization to 0.2 μM of each of the detection probes ID16, IDS3, or ID33 in a buffer containing 20% formamide, 2× SSC, and 5% glycerol for 30 min at 37 °C. The slides were washed in wash buffers 1 and 2, dehydrated, mounted with Vectashield and visualized as described previously.[49]

Depletion of Endogenous TopI from HEK293T Cell Extracts. TopI was depleted from nuclear extracts by incubating the extract from 1 × 10^7 HEK293T cells with 5 nmol of the biotinylated suicide substrate in a total volume of 70 μL containing 0.5 M NaCl, 20 mM HEPES, pH 7.9, and 20% glycerol for 30 min at room temperature (22−25 °C). Subsequently, cleavage complexes were removed by adding 20 μL of streptavidine-coupled magnetic beads essentially as described by the manufacturer. As a control, a mock depletion was carried out as described above except that suicide substrate was omitted from the reaction mixture. Depletion was tested by Western blotting essentially as described previously.[50]

Acknowledgment. We are thankful to medical technician S. Jacobsen for technical assistance during this project and to Dr. F. Stewart and Dr. K. Rajewsky for kindly providing the plasmids pCAGGA FLPe and pPGK Cre pA, respectively. Also we wish to thank Dr. M. Jayaram, for kindly providing pET11a(Flp) for expression and purification of recombinant Flp, and to Drs. A. Kwiatek and C.-H. Ma from M. Jayaram's laboratory for providing purified Cre for the investigations. This work was supported by grants from the John and Birthe Meyer Foundation, the Aase og Ejnar Danielsens Foundation, the Civilingenør Frode V. Nyegaard og Hustrus Foundation, the Carlsberg Foundation, the Danish Research Councils, the Danish Cancer Society, the Novo Nordisk Foundation, the Augustinus Foundations, the Hartmann Foundation, the Harboe Foundation, the Arvid Nilsson Foundation, the Direktør Einar Hansen og hustru fru Vera Hansen Foundation, the Fabrikant Einar Willumsen Foundation, the Fru Astrid Thaysen Foundation, the Karen Elise Jensen Foundation, and the Købmand Sven Hansen og hustru Ina Hansen's Foundation

REFERENCES AND NOTES

1. Belle, D. J.; Singh, H. Genetic Factors in Drug Metabolism. *Am. Fam. Physician* **2008**, *77*, 1553–1560.
2. Stougaard, M.; Lohmann, J. S.; Mancino, A.; Celik, S.; Andersen, F. F.; Koch, J.; Knudsen, B. R. Single-Molecule Detection of Human Topoisomerase I Cleavage−Ligation Activity. *ACS Nano* **2009**, *3*, 223–233.
3. Champoux, J. J. Mechanism of Catalysis by Eukaryotic DNA Topoisomerase I. *Adv. Pharmacol.* **1994**, *29A*, 71–82.
4. Pommier, Y. Topoisomerase I Inhibitors: Camptothecins and Beyond. *Nat. Rev. Cancer* **2006**, *6*, 789–802.
5. Liao, Z.; Robey, R. W.; Guirouilh-Barbat, J.; To, K. K.; Polgar, O.; Bates, S. E.; Pommier, Y. Reduced Expression of DNA Topoisomerase I in SF295 Human Glioblastoma Cells Selected for Resistance to Homocamptothecin and Diflomotecan. *Mol. Pharmacol.* **2008**, *73*, 490–497.
6. Soret, J.; Gabut, M.; Dupon, C.; Kohlhagen, G.; Stévenin, J.; Pommier, Y.; Tazi, J. Altered Serine/Arginine-Rich Protein Phosphorylation and Exonic Enhancer-Dependent Splicing in Mammalian Cells Lacking Topoisomerase I. *Cancer Res.* **2003**, *63*, 8203–8211.
7. Taniguchi, K.; Kohno, K.; Kawanami, K.; Wada, M.; Kanematsu, T.; Kuwano, M. Drug-Induced Down-Regulation of Topoisomerase I in Human Epidermoid Cancer Cells Resistant to Saintopin and Camptothecins. *Cancer Res.* **1996**, *56*, 2348–2354.
8. Fire, A.; Xu, S. Q. Rolling Replication of Short DNA Circles. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4641–4645.
9. Banér, J.; Nilsson, M.; Mendel-Hartvig, M.; Landegren, U. Signal Amplification of Padlock Probes by Rolling Circle Replication. *Nucleic Acids Res.* **1998**, *26*, 5073–5078.
10. Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification. *Nat. Genet.* **1998**, *9*, 225–232.
11. Lam, L.; Iino, R.; Tabata, K. V.; Noji, H. Highly Sensitive Restriction Enzyme Assay and Analysis: A Review. *Anal. Bioanal. Chem.* **2008**, *391*, 2423–2432.
12. Healy, K. Nanopore-Based Single-Molecule DNA Analysis. *Nanomedicine* **2007**, *2*, 459–481.
13. Charvin, G.; Strick, T. R.; Bensimon, D.; Croquette, V. Tracking Topoisomerase Activity at the Single-Molecule Level. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 201–219.
14. van Mameren, J.; Peterman, E. J.; Wuite, G. J. See Me, Feel Me: Methods to Concurrently Visualize and Manipulate Single DNA Molecules and Associated Proteins. *Nucleic Acids Res.* **2008**, *36*, 4381–4389.
15. Herbert, K. M.; Greenleaf, W. J.; Block, S. M. Single-Molecule Studies of RNA Polymerase: Motoring Along. *Annu. Rev. Biochem.* **2008**, *77*, 149–176.
16. Wang, L.; O'Donoghue, M. B.; Tan, W. Nanoparticles for Multiplex Diagnostics and Imaging. *Nanomedicine* **2006**, *1*, 413–426.
17. Hansen, S. G.; Frøhlich, R. F.; Knudsen, B. R. Type IB Topoisomerases and Tyrosine Recombinases—Distinct Functions within Related Structural Frameworks. *Curr. Top. Biochem. Res.* **2003**, *5*, 149–159.
18. Grainge, I.; Jayaram, M. The Integrase Family of Recombinase: Organization and Function of the Active Site. *Mol. Microbiol.* **1999**, *33*, 449–456.
19. Andrews, B. J.; Proteau, G. A.; Beatty, L. G.; Sadowski, P. D. The FLP Recombinase of the 2 Micron Circle DNA of Yeast: Interaction with its Target Sequences. *Cell.* **1985**, *40*, 795–803.
20. Abremski, K.; Hoess, R. Bacteriophage P1 Site-specific Recombination. Purification and Properties of the Cre Recombinase Protein. *J. Biol. Chem.* **1984**, *259*, 1509–1514.
21. García-Otín, A. L.; Guillou, F. Mammalian Genome Targeting Using Site-Specific Recombinases. *Front Biosci.* **2006**, *11*, 1108–1136.
22. Branda, C. S.; Dymecki, S. M. Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice. *Dev. Cell* **2004**, *6*, 7–28.
23. Bolusani, S.; Ma, C. H.; Paek, A.; Konieczka, J. H.; Jayaram, M.; Voziyanov, Y. Evolution of Variants of Yeast Site-Specific Recombinase Flp That Utilize Native Genomic Sequences as Recombination Target Sites. *Nucleic Acids Res.* **2006**, *34*, 5259–5269.
24. Konieczka, J. H.; Paek, A.; Jayaram, M.; Voziyanov, Y. Recombination of Hybrid Target Sites by Binary Combinations of Flp Variants: Mutations That Foster Interprotomer Collaboration and Enlarge Substrate Tolerance. *J. Mol. Biol.* **2004**, *339*, 365–378.
25. Voziyanov, Y.; Konieczka, J. H.; Stewart, A. F.; Jayaram, M. Stepwise Manipulation of DNA Specificity in Flp Recombinase: Progressively Adapting Flp to Individual and Combinatorial Mutations in its Target Site. *J. Mol. Biol.* **2003**, *326*, 65–76.
26. Akopian, A.; Marshall Stark, W. Site-Specific DNA Recombinases as Instruments for Genomic Surgery. *Adv Genet.* **2005**, *55*, 1–23.
27. Bonven, B. J.; Gocke, E.; Westergaard, O. A High Affinity Topoisomerase I Binding Sequence Is Clustered at DNAase I Hypersensitive Sites in Tetrahymena R-Chromatin. *Cell* **1985**, *41*, 541–551.
28. Lisby, M.; Krogh, B. O.; Boege, F.; Westergaard, O.; Knudsen, B. R. Camptothecins Inhibit the Utilization of Hydrogen Peroxide in the Ligation Step of Topoisomerase I Catalysis. *Biochemistry* **1998**, *37*, 10815–10827.
29. Andersen, A. H.; Christiansen, K.; Westergaard, O. Uncoupling of Topoisomerase-Mediated DNA Cleavage and Religation. *Methods Mol. Biol.* **2001**, *95*, 101–117.
30. Frøhlich, R. F.; Andersen, F. F.; Westergaard, O.; Andersen, A. H.; Knudsen, B. R. Regions within the N-Terminal Domain of Human Topoisomerase I Exert Important Functions during Strand Rotation and DNA Binding. *J. Mol. Biol.* **2004**, *336*, 93–103.
31. Amin, A.; Roca, H.; Luetke, K.; Sadowski, P. D. Synapsis, Strand Scission, and Strand Exchange Induced by the FLP

Recombinase: Analysis with Half-FRT Sites. *Mol. Cell. Biol.* **1991**, *11*, 4497–4508.
32. Qian, X. H.; Inman, R. B.; Cox, M. M. Reactions between Half- and Full-FLP Recombination Target Sites. A Model System for Analyzing Early Steps in FLP Protein-Mediated Site-Specific Recombination. *J. Biol. Chem.* **1992**, *267*, 7794–7805.
33. Mack, A.; Sauer, B.; Abremski, K.; Hoess, R. Stoichiometry of the Cre Recombinase Bound to the Lox Recombining Site. *Nucleic Acids Res.* **1992**, *20*, 4451–4455.
34. Christiansen, K.; Svejstrup, A. B.; Andersen, A. H.; Westergaard, O. Eukaryotic Topoisomerase I-Mediated Cleavage Requires Bipartite DNA Interaction. Cleavage of DNA Substrates Containing Strand Interruptions Implicates a Role for Topoisomerase I in Illegitimate Recombination. *J. Biol. Chem.* **1993**, *268*, 9690–9701.
35. Lehman, I. R. DNA Ligase: Structure, Mechanism, and Function. *Science* **1974**, *186*, 790–797.
36. Ceska, T. A.; Sayers, J. R. Structure-Specific DNA Cleavage by 5′ Nucleases. *Trends Biochem. Sci.* **1998**, *23*, 331–336.
37. Buchholz, F.; Angrand, P. O.; Stewart, A. F. Improved Properties of FLP Recombinase Evolved by Cycling Mutagenesis. *Nat. Biotechnol.* **1998**, *6*, 617–618.
38. Schaft, J.; Ashery-Padan, R.; van der Hoeven, F.; Gruss, P.; Stewart, A. F. Efficient FLP Recombination in Mouse ES Cells and Oocytes. *Genesis* **2001**, *31*, 6–10.
39. Soriano, P.; Montgomery, C.; Geske, R.; Bradley, A. Targeted Disruption of the c-src Proto-Oncogene Leads to Osteopetrosis in Mice. *Cell* **1991**, *64*, 693–702.
40. Kondo, S.; Takahashi, Y.; Shiozawa, S.; Ichise, H.; Yoshida, N.; Kanegae, Y.; Saito, I. Efficient Sequential Gene Regulation *via* FLP- and Cre-Recombinase Using Adenovirus Vector in Mammalian Cells Including Mouse ES Cells. *Microbiol. Immunol.* **2006**, *50*, 831–483.
41. Yamamoto, M.; Shook, N. A.; Kanisicak, O.; Yamamoto, S.; Wosczyna, M. N.; Camp, J. R.; Goldhamer, D. J. A Multifunctional Reporter Mouse Line for Cre- and FLP-dependent Lineage Analysis. *Genesis* **2009**, *47*, 107–114.
42. Rossant, J.; McMahon, A. "Cre"-ating Mouse Mutants—A Meeting Review on Conditional Mouse Genetics. *Genes Dev.* **1999**, *13*, 142–145.
43. Knudsen, B. R.; Straub, T.; Boege, F. Separation and Functional Analysis of Eukaryotic DNA Topoisomerases by Chromatography and Electrophoresis. *J. Chromatogr., B* **1996**, *684*, 307–321.
44. Prasad, P. V.; Young, L. J.; Jayaram, M. Mutations in the 2-Microns Circle Site-Specific Recombinase that Abolish Recombination without Affecting Substrate Recognition. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2189–2193 (Correction: *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1497.
45. Mo, Y. Y.; Wang, P.; Beck, W. T. Functional Expression of Human DNA Topoisomerase I and its Subcellular Localization in HeLa Cells. *Exp. Cell Res.* **2000**, *256*, 480–490.
46. Hann, C. L.; Carlberg, A. L.; Bjornsti, M. A. Intragenic Suppressors of Mutant DNA Topoisomerase I-induced Lethality Diminish Enzyme Binding of DNA. *J. Biol. Chem.* **1998**, *273*, 31519–31527.
47. Christiansen, K.; Knudsen, B. R.; Westergaard, O. The Covalent Eukaryotic Topoisomerase I-DNA Intermediate Catalyzes pH-Dependent Hydrolysis and Alcoholysis. *J. Biol. Chem.* **1994**, *269*, 11367–11373.
48. Andersen, F. F.; Andersen, K. E.; Kusk, M.; Frøhlich, R. F.; Westergaard, O.; Andersen, A. H.; Knudsen, B. R. Recombinogenic Flap Ligation Mediated by Human Topoisomerase I. *J. Mol. Biol.* **2003**, *330*, 235–246.
49. Stougaard, M.; Lohmann, J. S.; Zajac, M.; Hamilton-Dutoit, S.; Koch, J. *In Situ* Detection of Non-polyadenylated RNA Molecules Using Turtle Probes and Target Primed Rolling Circle PRINS. *BMC Biotechnol.* **2007**, *7*, 69.
50. Hede, M. S.; Petersen, R. L.; Frøhlich, R. F.; Krüger, D.; Andersen, F. F.; Andersen, A. H.; Knudsen, B. R. Resolution of Holliday Junction Substrates by Human Topoisomerase I. *J. Mol. Biol.* **2007**, *365*, 1076–1092.