Université de Sherbrooke

Caractérisation initiale de l'instabilité génétique des spermatides de mammifères

Par

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Département de Biochimie

Thèse présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du grade de philosophiae doctor (Ph.D.) en Biochimie

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Résumé

Cartographie des cassures d'ADN chez les spermatides

Par
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Thèse présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de philosophiae doctor (Ph.D.) en Biochimie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

La spermatogenèse est un processus complexe permettant la génération de gamètes mâles ultra spécialisés, les spermatozoïdes. Plusieurs réorganisations successives de l'ADN sont essentielles pour la génération de gamètes mâles haploïdes, dont l'enjambement durant la méiose. La dernière étape de la spermatogenèse, la spermiogenèse, comporte une importante réorganisation nucléaire accompagnée de nombreuses cassures bicaténaires d'ADN, ce qui pourrait mener à une instabilité génétique, surtout dans ce contexte haploïde vulnérable. Le premier objectif de mes recherches était de mieux caractériser cette étape de remodelage chromatinien. Par une approche d'immunofluorescence, nous avons démontré la présence de l'enzyme topoisomérase IIβ (TOP2B) lors du remodelage de la chromatine, ainsi qu'une réponse aux dommages à l'ADN coïncidant avec le remodelage chromatinien par l'apparition de la phosphorylation du variant d'histone H2AFX, une biomarqueur de cassures bicaténaires. Il est donc fort probable que les spermatides utilisent un système de réparation propice à l'erreur, tel que la jonction terminale non-homologue (NHEJ) pour réparer les nombreuses cassures observées à ces étapes, menant possiblement à une instabilité génomique importante.

Afin de mieux comprendre l'impact d'une réparation inadéquate ou d'une absence de réparation de ces cassures, nous avons voulu déterminer leur distribution sur le génome murin. Or, il n'existait aucune approche méthodologique permettant de cartographier ces cassures à l'échelle génomique. Utilisant plusieurs modèles *in vitro* et *in vivo*, nous avons mis au point une approche unique, appelée *damaged DNA immunoprecipitation* ou dDIP, pouvant enrichir les régions endommagée sans compromettre la résolution nucléotidique. Par la suite, nous avons mis au point une méthodologie de dDIP pour les cellules d'eucaryotes supérieurs en immobilisant les cellules dans une matrice d'agarose pour limiter l'introduction de dommages non-spécifiques.

Le remodelage de la chromatine des spermatides représente une étape d'instabilité génomique encore peu explorée et pourrait s'avérer une source insoupçonnée de diversité génétique. Grâce à la création de la nouvelle méthodologie dDIP, il sera maintenant possible d'explorer l'importance des cassures transitoires observées durant ce drastique changement nucléaire pour les générations futures. De plus, cet outil peut être appliqué à différents types de dommages, tels que les dommages causés par le rayonnement ultraviolet et les dommages oxydatifs, et donc être utilisé dans l'étude de l'instabilité génomique et de la réparation de l'ADN dans de nombreux domaines scientifiques comme le cancer, la sénescence et la toxicologie.

Mots-clés : spermiogenèse, cassure d'ADN, réparation d'ADN, chromatine, spermatide

Épigraphe

« Si une expérience semble facile, elle sera difficile. Si une expérience semble difficile, elle sera impossible. »

Dicton scientifique d'origine inconnue

« Le futur n'est jamais écrit à l'avance pour personne, votre futur sera exactement ce que vous en ferez alors faites qu'il soit beau pour chacun de vous. »

Emmet Brown, Ph.D., Back to the future (1985)

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Liste des abréviations

BSA : *bovine serum albumin*

CdS : spermatide condensée

CS : spermatide condensante

dDIP : damaged DNA immunoprecipitation

DOP-PCR : degenerate oligonucleotide primed PCR

DSBs : Cassures bicaténaires d'ADN

ES : spermatide allongeante

Indels : insertions et délétions

IP : immunoprécipitation

L : spermatocyte leptotène

LM-PCR : ligation-mediated PCR

NHEJ : jonction terminale non-homologue, *non-homologous end-joining*

P : spermatocyte pachytène

PCR : polymerase chain reaction

PRMs : protamines

TAM-PCR : Terminal transférase and adaptor-mediated PCR

qPCR : PCR en temps réel

ROS : espèces réactives d'oxygène

S : cellule de Sertoli

SSBs : cassures moncaténaires d'ADN

TD-PCR : terminal transférase-dependent PCR

TdT : Terminal désoxynucleotidyl transférase

TPs : Protéines de transition

Z : spermatocyte zygotène

Introduction

Les testicules, siège de la spermatogenèse

Les testicules constituent le siège de la formation de gamètes mâles chez les mammifères et sont aussi responsables de la synthèse de plusieurs stéroïdes (Hermo, Pelletier, Cyr, & Smith, 2010). Afin de permettre une gamétogenèse efficace, leur température est généralement maintenue légèrement inférieure à celle du corps grâce à une extériorisation ajustable (Setchell, 1998). Cette thermorégulation est critique car, chez l'humain, des températures scrotales élevées causées par de la fièvre ou certaines habitudes de vie sont corrélées avec une diminution importante de la qualité des gâmètes (Jung & Schuppe, 2007).

La spermatogenèse chez les mammifères est un processus élaboré et finement régulé par lequel une spermatogonie, la cellule souche germinale, se différencie en spermatozoïde. Les tubules séminifères, contenus dans la capsule appelée *tunica albuginea*, permettent le développement et la maturation des cellules germinales, qui se déplacent de la membrane basale vers la lumière du tubule séminifère où les spermatozoïdes sont libérés et migrent vers l'épididyme(Loonie D Russell, 1990).

Les cellules de Sertoli

Les cellules de Sertoli, celulles nourissières des cellules germinales, occupent l'espace allant de la membrane basale du tubule seminifère jusqu'à sa lumière, créant une structure organisée et stratifiée. Elles assurent le bon déroulement de la spermatogenèse et régulent la sécrétion du fluide dans les tubules séminifères (Griswold, 1998). Les cellules de Sertoli sont interconnectées par plusieurs types de jonctions, créant une barrière unique entre les vaisseaux sanguins et les cellules

germinales, appelée la barrière hématotesticulaire, empêchant de développer une autoimmunité envers les spermatozoïdes et permettant le contrôle la diffusion de nombreuses molécules(Cheng & Mruk, 2002; Dym & Fawcett, 1970; Mital, Hinton, & Dufour, 2011; Setchell, 1969).

Les cellules de Leydig

Les cellules de Leydig sont la principale source d'androgènes chez les males et elles sont situées dans l'espace interstitial entre les tubules séminifères. L'hormone lutéinisante stimule la production d'androgènes, dont la testostérone qui est essentielle à la progression de la spermatogenèse (Hermo et al., 2010).

La spermatogenèse

Historiquement, on divise la spermatogenèse en trois parties successives : la spermatocytogenèse, la spermatidogenèse et la spermiogenèse (voir Figure 1). Durant la spermatocytogenèse, les spermatogonies renouvellent leur pool et s'engagent dans la voie germinale en se différenciant en spermatocyte qui se divisent lors de la méiose I en spermatocytes secondaires. Ceux-ci se divisent à nouveau lors de la méiose II dans un court laps de temps pour donner les spermatides, ce qui constitue la spermatidogenèse. Lors de la spermiogenèse, les spermatides rondes se transforment de façon incrémentale en gamètes mâles matures. Cette métamorphose des spermatides entrainera la formation de l'acrosome et du flagelle, une régulation unique de la synthèse et de l'entreposage des ARN messagers, une réorganisation majeure de ses organelles, un remodelage de la chromatine, une compaction drastique du noyau et la perte presque complète du cytoplasme.

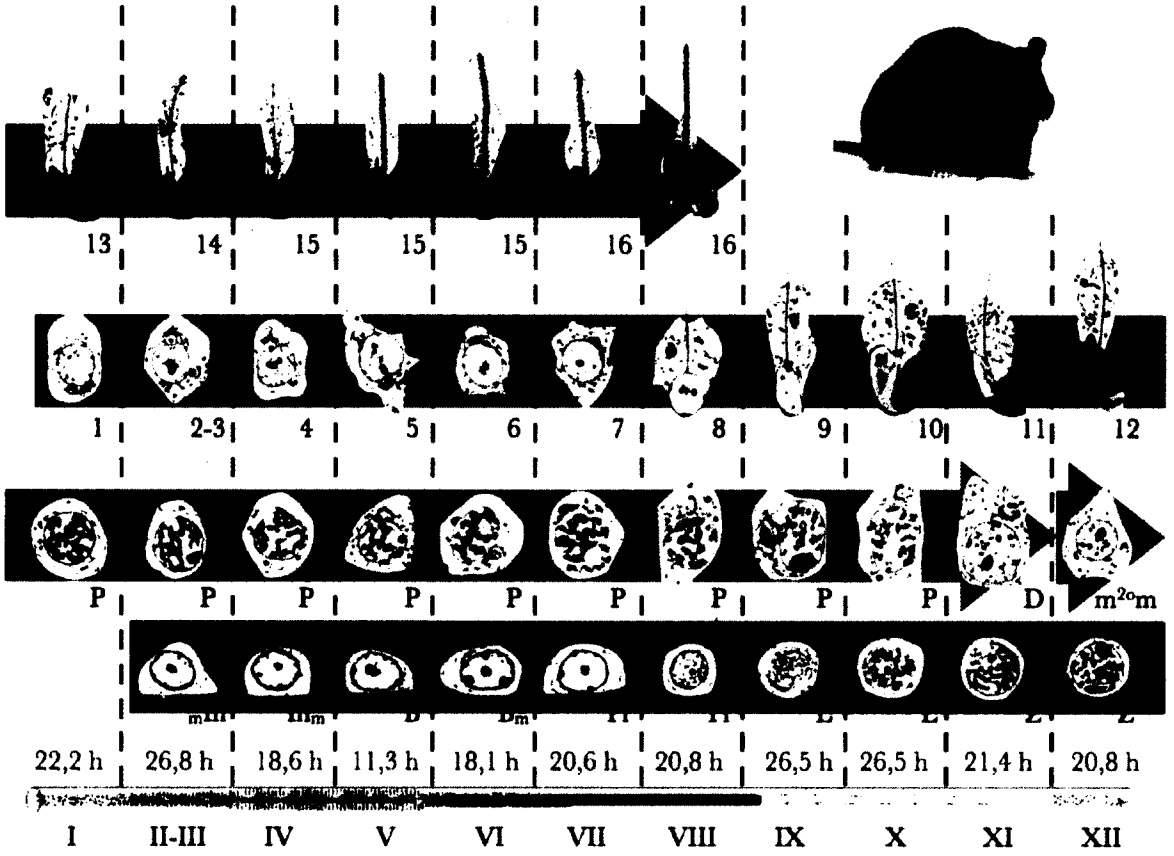

Figure 1. Stades de la spermatogenèse chez la souris.
La spermatogenèse murine est divisée en trois parties, soit la spermatocytogenèse (en vert), la spermatidogenèse (en rouge) et la spermiogenèse (en bleu). Cette figure démontre aussi le patron de transillumination des tubules séminifères associé à chacun des stades, ainsi que la durée de chaque stade *in vivo*. Cette figure a été adaptée à partir de deux ouvrages(Dee Russell, 1990; Kotaja et al., 2004).

L'infertilité masculine

Au Canada, la prévalence de l'infertilité en 2009-2010 se situait entre 11,5 et 15,7% (Bushnik, Cook, Yuzpe, Tough, & Collins, 2012). Généralement, on attibue la moitié des cas d'infertilité à l'homme, dont seulement 15% ont une éthiologie établie (Rajender, Avery, & Agarwal, 2011). Bien que l'apparence, la motilité et le nombre de spermatozoïdes sont les critères utilisés par l'Organisation Mondiale de la Santé (OMS) (Virro, Larson-Cook, & Evenson, 2004), des études récentes ont démontré que la fragmentation de l'ADN et la qualité de la compaction de la chromatine des

spermatozoïdes sont beacoup plus prédictifs de la fertilité d'un homme (Fatehi et al., 2006; Gorczyca, Traganos, Jesionowska, & Darzynkiewicz, 1993; Irvine et al., 2000; Manicardi et al., 1995; Muratori et al., 2000; Simon et al., n.d.). De plus, l'altération de la compaction du génome paternel a aussi été associée avec l'apparition de maladies génétiques *de novo*, des défauts dévelopementaux et morphologiques, le cancer et des avortements spontannés (Emery & Carrell, 2006; Kleinhaus et al., 2006; Marchetti & Wyrobek, 2005; Tesarik, Greco, & Mendoza, 2004). La fragmentation de l'ADN des spermatozoïdes peut aussi compromettre l'utilisation de technologies de reproduciton assistée (Tesarik et al., 2004). Il est donc essentiel de mieux comprendre les mécanismes qui peuvent mener à l'infertilité masculine pour proposer de meilleures avenues de traitement.

Bien que plusieurs outils moléculaires ont été développés pour mesurer adéquatement ces paramètres en clinique [pour une revue sur le sujet, consultez (Evenson & Wixon, 2006)], nous avons peu de pistes concernant l'origine de la fragmentation de l'ADN des spermatozoïdes et de la compaction de celui-ci. Au cours des années, plusieurs chercheurs ont proposé différentes hypothèses pouvant expliquer les cas inexpliqués d'infertilité masculine. Parmi celles-ci, nous retrouvons l'apoptose abortive, les espèces réactives de l'oxygène et le remodelage de la chromatine des spermatides.

L'apoptose abortive

Durant la spermatogenèse, l'apoptose est essentielle pour maintenir un ratio optimal entre les cellules de Sertoli et les cellules germinales. De plus, il existe des points de contrôle qui permettent l'élimination des cellules dont le développement est abérent, notamment chez les spermatocytes pachytènes. Sakkas et collègues ont alors suggéré que le taux de fragmentation important de l'ADN des spermatozoïdes de certains patients infertiles pourrait être le résultat d'une apoptose incomplète (Sakkas et al., 1999). Cette hypothèse, aussi appelée apoptose abortive des spermatides, fut renforcée par l'observation chez les spermatides de composantes s'apparentant à

l'apoptose, telles que la présense de la protéine Fas, des défauts structurels des noyaux fragmentés, des cytoplasmes avec des organelles aggrégées, l'altération de l'intégrité des mitochondries, la présence de Bcl-x, des caspases et de p53 (Baccetti et al., 1997; Donnelly, O'Connell, McClure, & Lewis, 2000; Gandini et al., 2000; Sakkas et al., 2002; Weng et al., 2002). Par contre, aucune évidence n'a été présentée jusqu'à présent qui permettrait de faire le lien entre ces observations et la fragmentation de l'ADN mesurée par la technique TUNEL (Muratori, Marchiani, Maggi, Forti, & Baldi, 2006). Or, contrairement aux premières observations publiées supportant cette hypothèse, la fragmentation de l'ADN chez les spermatides n'est pas restreinte à une sous-population, mais bien à l'ensemble des spermatides, tel que démontré par notre laboratoire (Laberge & Boissonneault, 2005; Marcon & Boissonneault, 2004), démontrant un programme normal de la spermiogenèse impliquant une phase transitoire de cassures de l'ADN. Ces résultats seront davantage discutés dans les prochaines sections de l'introduction.

Au cours des dernières années, l'hypothèse de l'apoptose abortive a été mise de côté par manque d'obervations la supportant. De plus, il a été démontré que les spermatides sont résistants à l'apoptose lorsqu'irradiés par rayonnement gamma, ce qui indique qu'elles ne possèderaient pas la machinerie cellulaire ni les points de contrôle nécessaires pour activer un tel processus (Ahmed, de Boer, Philippens, Kal, & de Rooij, 2010; OAKBERG & DIMINNO, 1960).

Les espèces réactives de l'oxygène

La présence d'espèces réactives de l'oxygène (ou ROS, *reactive oxygen species*) est associée avec une perte importante de la motilité et du potentiel fertilisant, ainsi qu'avec la présence de dommages à l'ADN (Aitken & Krausz, 2001; Aitken & de Iuliis, 2009; O, Chen, & Chow, 2006). La sécretion de molécules ou enzymes antioxydantes protège les spermatozoïdes contre les ROS, mais des dommages peuvent survenir si l'équilibre est débalancé par des causes endogènes ou exogènes (des xénobiotiques,

par exemple) (O et al., 2006). L'amélioration de la fertilité grâce à la prescription de régimes d'antioxydants aux hommes infertiles est encore controversée, mais semble procurer une certaine protection et des résultats améliorés par rapport à certaines techniques de fertilisation *in vitro* (Agarwal, Nallella, Allamaneni, & Said, 2004; Greco, 2005).

Les spermatozoïdes humains pourraient être potentiellement plus susceptibles aux attaques des ROS car ils possèdent davantage d'histones résiduelles, soit 10 à 20% comparé à d'autres mammifères tels que la souris où l'on peut observer généralement 1-4% (Dadoune, 2003; van der Heijden et al., 2006; Wykes & Krawetz, 2003b). Ainsi, les ROS représentent une hypothèse crédible et supportée pour expliquer certains cas d'infertilité masculine.

Le remodelage de la chromatine des spermatides

Parmi les hypothèses possibles de l'origine de la fragmentation de l'ADN des spermatozoïdes, le spectaculaire remodelage de la chromatine des spermatides comporte plusieurs éléments pouvant mener aux paramètres observés chez certains patients infertiles : une génération de cassures d'ADN transitoires, des systèmes limités de réparation de l'ADN et aucun point de contrôle connu.

Au début de mes travaux de doctorat, très peu était connu du remodelage de la chromatine des spermatides. Grâce à ce mécanisme unique chez les eucaryotes, le génome est condensé dix fois plus que chez les cellules somatiques et donne au noyau une forme plus hydrodynamique. Pour atteindre ces niveaux de compaction, les histones sont successivement remplacées par les protéines de transition (TPs), puis finalement par de petites protéines basiques riches en arginine, les protamines (PRMs). Ainsi, les protamines, hautement chargées positivement, neutralisent efficacement le phosphosquelette de l'ADN et permettent un rapprochement accru des brins d'ADN, procurant ainsi au gamète mâle une plus importante compaction du

matériel génétique (voir Figure 2). Marquant le début de ce changement majeur au niveau de la chromatine, les extrémités N-terminales des histones H4 sont acétylées massivement, permettant le relâchement de la chromatine histonique et facilitant ainsi l'échange avec les protéines de transition (Christensen, Rattner, & Dixon, 1984; Marcon & Boissonneault, 2004; Oliva & Mezquita, 1982). En plus des modifications post-traductionnelles des histones canoniques observées récemment (Baarends et al., 1999; H. Y. Chen, Sun, Zhang, Davie, & Meistrich, 1998), l'incorporation de nombreux variants d'histones spécifiques au testicule au cours des premières étapes de la spermiogenèse contribue sans aucun doute à cette transition majeure de la chromatine (Churikov et al., 2004; Govin et al., 2007; Martianov et al., 2005).

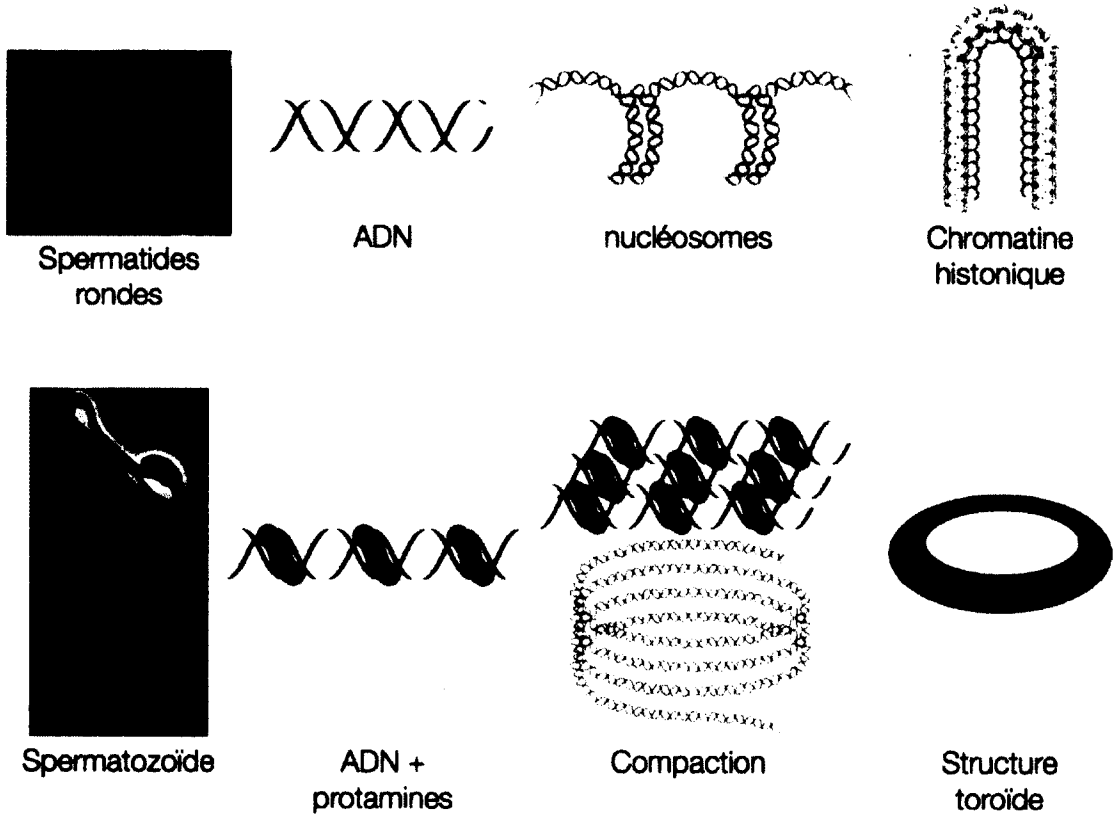

Figure 2. Organisation de la chromatine et topologie de l'ADN des spermatides rondes et des spermatozoïdes.

Un remodelage incomplet et structure chromatinienne des spermatozoïdes

Bien que la grande majorité des histones soient remplacées, la protamination n'est pas complète et varie d'une espèce à l'autre ; chez la souris, les spermatozoïdes conservent 1-4 % d'histones, alors que, chez l'humain, cela peut atteindre 20 % (Dadoune, 2003; Gatewood, Cook, Balhorn, Schmid, & Bradbury, 1990; Gusse et al., 1986; Tanphaichitr, Sobhon, Taluppeth, & Chalermisarachai, 1978; Wykes & Krawetz, 2003b). Le modèle de la structure chromatinienne des spermatozoïdes proposé par Dr Steven Ward décrit une organisation majoritaire de toroïdes protaminés englobant environ 50 000 paires de bases et quelques régions conservant une chromatine histonique; ces domaines génomiques seraient joints par de courtes régions sensibles à une digestion à la DNase I, appelée régions d'attachement à la matrice nucléaire ou MARs (*matrix attachment regions*)(Ward, 2010). Plusieurs hypothèses ont été formulées pour expliquer la rétention de régions histoniques chez les spermatozoïdes. La protamination incomplète pourrait expliquer ce phénomène, mais prendrait pour acquis que les régions de protamination incomplète ne soient pas associées avec des séquences spécifiques (Gatewood, Cook, Balhorn, Bradbury, & Schmid, 1987). Or, plusieurs études démontrent une rétention préférentielle des histones à des loci spécifiques. Il semble que deux explications complémentaires émergent des différentes études, soit la rétention des histones associée à la transcription active chez les spermatides et à la transcription future chez l'embryon.

Utilisant comme modèle le locus des protamine 1 et 2 (PRM1 et PRM2), ainsi que de la protéine de transition 2 (TNP2), Wykes et Krawetz ont démontré la rétention des histones sur ce locus grandement transcrit durant la spermiogenèse et cerné par deux sites d'attachement à la matrice nucléaire (Wykes & Krawetz, 2003b). Plus récemment et grâce au perfectionnement des certaines technologies génomiques, telles que les micropuces et le séquençage de deuxième génération, il a été possible d'établir une carte génomique des séquences préférencielles pour la rétention des histones. Deux études indépendantes ont démontré clairement le caractère non-aléatoire de la rétention des histones particulièrement au niveau des promoteurs de certaines

familles de gènes exprimés rapidement chez l'embryon (Arpanahi et al., 2009; Hammoud et al., 2009). Ces résultats suggèrent que la rétention des histones permettrait la transmission d'une information épigénétique importante à la prochaine génération. Pour une revue de littérature plus approfondie sur la structure de la chromatine du spermatozoïde et la rétention des histones, veuillez consulter l'Annexe 1 (Johnson et al., 2011), ainsi que le chapitre de livre suivant (Grégoire, Leduc, & Boissonneault, 2011).

L'hypothèse de départ

Les travaux de deux étudiants précédents dans mon laboratoire d'accueil ont permis de confirmer l'hyperacétylation de l'histone H4 chez les spermatides murins et de mettre en évidence des cassures d'ADN transitoires accompagnant le début du remodelage de la chromatine (Laberge & Boissonneault, 2005; Marcon & Boissonneault, 2004). Ce phénomène avait déjà été rapporté mais chez une portion limitée de spermatides (McPherson & Longo, 1992; 1993). Ces travaux démontraient pour la première fois que ces cassures d'ADN faisaient partie intégrante du programme de différentiation normale des spermatides, chez la souris comme chez l'humain. De plus, les travaux subséquents ont démontré que ces cassures d'ADN étaient majoritairement bicaténaires en visualisant l'intégrité de l'ADN des spermatides en utilisant la technique COMET (*single cell electrophoresis*) et que la présence des cassures pouvaient être abolies par une incubation des spermatides avec deux inhibiteurs de topoisomérase de type II.

Ces résultats suggéraient alors une implication cruciale des topoisomérases de type II, mais aussi une activité catalytique anormale de celles-ci dans ce processus de remodelage de la chromatine. Cette observation était plus qu'inattendue puisque la réparation des cassures bicaténaires d'ADN par des cellules haploïdes comme les spermatides s'avère plus difficile et surtout moins fidèle car la réparation d'ADN de type « recombinaison homologue » est impossible, due à l'absence de chromatides

sœurs (voir le Tableau 1 pour un résumé des systèmes de réparation de l'ADN). Ceci va donc à l'encontre de la notion de préservation de l'intégrité du génome pour la transmission à la prochaine génération. Pour une revue plus approfondie des systèmes de réparation d'ADN chez les spermatides, veuillez consulter le chapitre de livre intitulé « *Post-meiotic DNA damage and response in male germ cells* » retrouvé à l'Annexe 2. Plusieurs articles suggéraient un rôle important des topoisomérases durant la spermiogenèse (J. L. Chen & Longo, 1996; McPherson & Longo, 1992; 1993; Roca & Mezquita, 1989). L'hypothèse suggérée par notre laboratoire pour expliquer ce phénomène se base sur la structure et la topologie de l'ADN chez le spermatozoïde (Boissonneault, 2002). Dans le noyau des spermatozoïdes, la topologie de l'ADN est plutôt linéaire grâce à l'incorporation des protamines dans les sillons de la double-hélice d'ADN et se circonscrit en structure toroïde tel qu'illustré à la Figure 2, contrairement à une topologie dite « somatique » où l'ADN est super-enroulé autour des nucléosomes constitués d'histone (Ward, 1993).

Tableau 1. Système de réparations d'ADN de cassures bicaténaires des cellules eucaryotes

Voies de réparation d'ADN	Protéines impliquées	Erreurs typiques
Recombinaison homologue (HR)	BRCA1/2, Rad51, XRCC2, LIG1, RPA	Sans erreur
Single strand annealing (SSA)	ERCC1, ERCC4 (XPF), Rad52	Indels (++) Translocation chromosomique (++)
Jonction terminale non-homologue dépendante de DNA PK (D-NHEJ)	DNA PKcs, Ku70, ARTEMIS, XRCC4, LIG4, XLF (NHEJ1)	Indels (+) Translocation chromosomique (+)
Jonction terminale non-homologue alternative (B-NHEJ)	PARP, XRCC1, LIG3	Indels (+) Translocation chromosomique (+)

+, occasionnel; ++, fréquent; (Ciccia & Elledge, 2010; Griffin & Thacker, 2004)

Ainsi, durant le remodelage de la chromatine, la spermatide doit éliminer les super-tours d'ADN lors de l'éjection des histones pour assurer une compaction maximale par

les protamines. Chez les cellules somatiques, les topoisomérases de type I et de type II peuvent modifier la topologie de l'ADN pour réduire les super-tours en créant une cassure monocaténaire ou bicaténaire respectivement pour permettre ce changement (Forterre, Gribaldo, Gadelle, & Serre, 2007). Les topoisomérases constituent donc des candidats logiques pour effectuer cette tâche chez les spermatides. Selon les évidences directes et indirectes précédant mes travaux, les deux isoformes de topoisomérase de type II chez les mammifères, soit la topoisomérase IIα (TOP2A) ou la topoisomérase IIβ (TOP2B), possédaient le profil idéal, c'est-à-dire une enzyme générant des cassures bicaténaires transitoires, ayant une activité enzymatique rapide, présent durant la spermatogenèse et pouvant être inhibée par l'étoposide et la suramine, deux inhibiteurs connus de la topoisoméase de type II (Laberge & Boissonneault, 2005). Mon hypothèse de départ stipulait que les topoisomérases de type II entrainaient des cassures d'ADN bicaténaires transitoires durant le remodelage de la chromatine des spermatides pour éliminer les super-tours de l'ADN pour assurer une compaction optimale du génome paternel par les protamines.

L'importance de ce projet de recherche

Le caractère haploïde et l'amplitude du remodelage de la chromatine rendent les spermatides particulièrement vulnérables à des atteintes à l'intégrité de leur génome; toute séquence d'ADN endommagée persistante ou dont la réparation est inexacte peut mener à des conséquences graves chez l'embryon issu de cette gamète, allant de l'apparition de maladies *de novo* à l'incompatibilité avec la vie (consultez l'Annexe 3 pour une revue en profondeur des conséquences possibles). D'une part, ce processus possiblement mutagène peut mener à une catastrophe génétique, mais d'autre part, il représente peut-être un processus encore inconnu d'évolution d'origine paternelle. De plus, une grande proportion d'hommes infertiles démontrent un plus haut niveau de fragmentation d'ADN au niveau de leur sperme, ayant possiblement pour origine la persistance des cassures d'ADN liées au remodelage des spermatides (Leduc, Nkoma,

& Boissonneault, 2008), dernière étape de la spermatogenèse dont on ne connait pas de point de contrôle (check point), s'il en existe.

Il est donc essentiel de mieux comprendre le mécanisme de remodelage de la chromatine des spermatides et de ses cassures d'ADN transitoires associées, car ils pourraient expliquer de nombreux cas idiopathiques d'infertilité masculine et mener vers de nouvelles avenues de traitements. De plus, il est crucial d'établir une carte à l'échelle du génome des cassures bicaténaires engendrées durant la spermiogenèse, afin de mieux comprendre leur importance pour l'embryon si elles persistent ou si elles sont réparées inadéquatement.

Les objectifs

Au cours de mes travaux de doctorat, j'ai eu pour objectifs de:

1) Caractériser davantage le remodelage de la chromatide et la réparation d'ADN des spermatides chez la souris,
2) Mettre au point une nouvelle méthode pour cartographier les cassures bicaténaires d'ADN en utilisant des modèles *in vitro* et *in vivo*, et
3) Cartographier les cassures bicaténaires des spermatides à l'aide de la technique dDIP et du séquençage de 2ème génération.

Chapitre 1 : Réponse aux dommages à l'ADN durant le remodelage de la chromatine des spermatides allongeantes de souris

DNA damage response during chromatin remodeling in elongating spermatids of mice

Frédéric Leduc, Vincent Maquennehan, Geneviève Bikond Nkoma et Guylain Boissonneault

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Avant-propos

Cet article s'inscrit dans les efforts déployés par le laboratoire du Professeur Guylain Boissonneault pour une meilleure compréhension des mécanismes impliqués lors du remodelage de la chromatine durant la spermiogenèse murine, une étape critique de la genèse de gamète mâle. Les prémisses de cet ouvrage s'appuient largement sur les résultats obtenus par des travaux publiés précédemment(Laberge & Boissonneault, 2005; Marcon & Boissonneault, 2004). Cet article présente les premières évidences directes de l'implication de la topoisomérase IIβ durant le remodelage de la chromatine des spermatides, ainsi que la présence d'une signalisation et une réparation active des cassures d'ADN.

J'ai a conçu les expériences, a effectué la majorité des expérimentations, a analysé et compilé les résultats et a rédigé l'article scientifique. Vincent Maquennehan, alors stagiaire COOP dans le laboratoire, a contribué à la mise au point de certaines méthodes et à la réalisation de quelques expériences. Geneviève Bikond Nkoma a participé à la mise au point de certaines approches et a contribué à l'analyse des résultats. Professeur Guylain Boissonneault a guidé la démarche expérimentale proposée par Frédéric Leduc et a contribué à la rédaction de l'article scientifique.

Résumé

Un empaquetage précis du génome paternel durant la spermiogenèse est essentiel pour la fertilisation et l'embryogenèse. La majorité du super-enroulement de l'ADN nucléosomal doit être éliminé chez les spermatides allongeantes (ES), et des cassures transitoires de l'ADN sont observées pour faciliter le processus. Les topoisomérases ont été considérées comme des candidates idéales pour l'élimination des super-tours de l'ADN, cependant, leur activité catalytique, dans le contexte d'un aussi important remodelage chromatinien, représente un risque important pour l'intégrité génomique. Par immunofluorescence, nous avons confirmé que la topoisomérase II bêta (TOP2B) est la topoisomérase de type II présente chez les ES entre les étapes 9 et 13. Il est intéressant de noter que la détection de TOP2B coïncide avec la détection de la tyrosyl-DNA phosphodiesterase 1 (TDP1), une enzyme connue pour résoudre des dommages à l'ADN médiés par les topoisomérases. La présence de gamma-H2AX (aussi appelé gamma-H2AFX) coïncidant avec les cassures d'ADN a aussi été confirmée à ces étapes et indique qu'une réponse aux dommages à l'ADN est déclenchée. Une réparation active de l'ADN a été démontrée par un essai fluorescent *in situ* de l'activité de l'ADN polymérase sur des préparations « écrasées » de tubules de stades choisis. Dans le contexte des spermatides haploïdes, toute cassure bicaténaire non résolue, provenant d'une défaillance dans le processus de ligation de la TOP2B, dépend probablement sur la recombinaison de jonction terminale non-homologue (NHEJ) pour sa réparation, un système prompt à l'erreur, car la recombinaison homologue est impossible en absence de chromatide sœur. Puisque ce processus de cassures transitoires fait partie intégrante du programme développemental normal des spermatides, des conséquences dramatiques pour l'intégrité génomique du gamète mâle en formation pourrait se produire advenant une quelconque altération de ce processus.

Abstract

A precise packaging of the paternal genome during spermiogenesis is essential for fertilization and embryogenesis. Most of the nucleosomal DNA supercoiling must be eliminated in elongating spermatids (ES), and transient DNA strand breaks are observed that facilitate the process. Topoisomerases have been considered as ideal candidates for the removal of DNA supercoiling, but their catalytic activity, in the context of such a major chromatin remodeling, entails genetic risks. Using immunofluorescence, we confirmed that topoisomerase II beta (TOP2B) is the type II topoisomerase present in ES between steps 9 and 13. Interestingly, the detection of TOP2B was found coincident with detection of tyrosyl-DNA phosphodiesterase 1 (TDP1), an enzyme known to resolve topoisomerase-mediated DNA damage. The presence of gamma-H2AX (also known as H2AFX) coincident with DNA strand breakage was also confirmed at these steps and indicates that a DNA damage response is triggered. Active DNA repair in ES was demonstrated using a fluorescent in situ DNA polymerase activity assay on squash preparations of staged tubules. In the context of haploid spermatids, any unresolved double-strand breaks, resulting from a failure in the rejoining process of TOP2B, must likely rely on the error-prone nonhomologous end joining, because homologous recombination cannot proceed in the absence of a sister chromatid. Because this process is part of the normal developmental program of the spermatids, dramatic consequences for the genomic integrity of the developing male gamete may arise should any alteration in the process occur.

Introduction

The precise packaging of the paternal genome during spermiogenesis produces a compact and more hydrodynamic nucleus and is essential for fertilization and embryogenesis [1- 3]. During the chromatin remodeling process in

spermatids, the necessary removal of most somatic histones is facilitated by the incorporation of histone variants [4, 5] as well as by massive posttranslational modifications of core histones, including ubiquitination [6, 7], sumoylation [8], and hyperacetylation of histones H3 and H4 [9, 10]. During this process, most of the nucleosomal DNA supercoiling must be eliminated [11]. One hypothesis is that such a drastic change in DNA topology must require DNA strand breaks to provide the necessary swivel to relieve torsional stress [12]. Not surprisingly, we observed that all elongating spermatids (ES) between steps 9 and 12 in the mouse harbor DNA fragmentation, as shown by TUNEL.

Therefore, DNA fragmentation is part of their normal differentiation program [13]. Topoisomerases have been considered as ideal candidates for the removal of DNA supercoiling in spermatids [12, 14- 17]. Type II topoisomerases are involved in a variety of DNA transactions, including transcription, DNA replication, chromatid separation, and chromosome condensation [18]. Topoisomerase II alters DNA topology through a double-strand break (DSB) and its subsequent religation [18]. Two closely related isoforms have been identified in human cells: topoisomerase IIα (TOP2A; 170 kDa) and topoisomerase IIβ (TOP2B; 180 kDa) [19]. Although they share high homology in their primary sequences, the two isoforms play different roles [20] and are differentially regulated during the cell cycle [21].

We and others provided evidence that a type II topoisomerase is involved in DNA fragmentation [14, 22, 23]. TUNEL labeling was greatly diminished in the presence of etoposide and suramin, two topoisomerase II inhibitors [24]. In addition, results from single-cell gel electrophoresis (COMET) of ES suggested that a majority of DNA strand breaks of normal ES were double stranded [24]. Unresolved DSBs resulting from a failure in the rejoining process by the topoisomerase II can have dramatic consequences on the genomic integrity of the developing male gamete, as these haploid cells may not rely on sister chromatids

for homologous recombination-based repair. Therefore, it is crucial to investigate any evidence of impaired topoisomerase II activity and whether any signature of DNA damage response (DDR) can be found in these cells. Should this be the case, one may consider the chromatin-remodeling steps as an important source of genetic instability that may have been overlooked.

Materials and methods

Animals

Seven-week-old male CD-1 mice were obtained from Charles River Breeding Laboratory (St-Constant, QC, Canada), maintained under standard housing conditions, and killed by CO_2 asphyxiation. Animal care was in accordance with the Université de Sherbrooke Animal Care and Use Committee.

Paraffin-Embedded Sections

Testes were excised, decapsulated, and fixed directly in Bouin solution (Sigma-Aldrich, St. Louis, MO) at 4°C for 24–48 h and then embedded in paraffin according to standard procedures. Sections (5 μm thick) were mounted on Superfrost Plus glass slides (Fisher Scientific, St-Laurent, QC, Canada).

Antibodies

The rabbit polyclonal anti-γH2AX (also known as H2AFX; catalog #ab2893) and rabbit polyclonal anti-TDP1 (tyrosyl-DNA phosphodiesterase 1; catalog #ab4166) were obtained from Abcam (Cambridge, MA) and diluted 1:100 for immunocytochemistry. The rabbit polyclonal anti-TOP2B antibody was purchased

from Santa Cruz Biotechnology (Santa Cruz, CA; catalog #sc- 13059) and diluted 1:25, whereas the anti-TOP2A antibody was obtained from TopoGEN (Port Orange, FL; catalog #2011-1) and used at a 1:100 dilution. Rhodamine or fluorescein coupled goat anti-rabbit IgG (H + L) minimal cross-reactivity antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA; catalog nos. 111-025-045 and 111-095-045, respectively). Nonspecific binding was never observed when these secondary antibodies were incubated alone.

Antigen Retrieval and Immunofluorescence on Sections

All incubations took place in a humidified chamber. Paraffin-embedded sections were deparaffinized as previously described [13]. Heat-induced epitope retrieval (HIER) was achieved by immersing slides in a boiling solution of 10 mM Tris and 1 mM EDTA (pH 9) for 5–10 min. The slides were cooled in running tap water for 10 min and then blocked with PBS containing 1.5% BSA and 0.5% Triton X-100 for 60 min at 37°C. Primary antibodies diluted in blocking solution were incubated for 60 min at 37°C. Slides were then washed three times with PBS containing 0.1% Triton X-100 (PBST) and incubated with secondary antibodies coupled with either fluorescein or rhodamine (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. Slides were washed with PBST, and DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or TO-PRO3 (Invitrogen Canada, Burlington, ON, Canada) and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

TUNEL and Immunofluorescence on Sections

Paraffin-embedded sections were deparaffinized, and HIER was carried out as outlined above. Slides were permeabilized with blocking solution for 60 min at 37°C and incubated with TUNEL labeling mix (Roche Diagnostics, Laval, QC,

Canada) containing 5 mM of $CoCl_2$, 0.1% Triton X-100, and 0.5 nmol of dATP-Biotin (Invitrogen Canada). Four hundred units of terminal transferase as well as a 1:100 dilution of the anti-γH2AX antibody were added, and the slides were incubated in a humidified chamber for 1 h at 37°C. Slides were washed with PBST and incubated in PBS containing diluted (1:100) streptavidin-FITC (Vector Laboratories) and diluted (1:200) rhodamine-coupled secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. Slides were washed with PBST, counterstained with DAPI, and mounted with Vectashield.

Collagenase Digestion and Squash Preparation

Testes were excised, decapsulated, and placed in a 50-ml flask containing 25 ml of freshly made 0.25 mg/ml collagenase (CLS-1; Worthington, Lakewood, NJ) in warm RPMI 1640 culture medium (Wisent, St-Bruno, QC, Canada). Testes were incubated in a shaking water bath at 32°C for 15 min or until the tubules were sufficiently dispersed. The tubules were then allowed to sediment, and the supernatant was discarded. The tubules were washed twice with 25 ml of warm RPMI.

Squash preparations were done as described by Kotaja et al. [25] with modifications. Under a transilluminating dissection microscope, 2-mm pieces of tubules were cut according to their stage-specific light-absorption pattern and transferred in 40 µl of 100 mM sucrose onto a Superfrost Plus slide. A coverslip was carefully placed over the tubule, and filter papers were used to remove excess fluid and to spread the cells out of the tubule. The slide was flash-frozen in liquid nitrogen for 20 sec, the coverslip was removed, and the cells were air-dried for 10 min at room temperature. The slides were used immediately.

In Situ Endogenous DNA Polymerase Assay

In situ endogenous DNA polymerase assays were carried out as described by Hecht and Parvinen [26] with modifications. Squash slides were incubated with TUNEL reaction mix (Roche Diagnostics) containing 5 mM of $CoCl_2$, 0.1% Triton X-100, and 1 nmol of dUTP-FITC (Enzo Life Sciences, Inc., Farmingdale, NY) without (endogenous DNA polymerase) or with (TUNEL) 400 U of terminal transferase (Roche Diagnostics) in a humidified chamber for 2 h at 30°C. As a negative control, 5 μl of 200 mM of EDTA (pH 9) were added in the endogenous DNA polymerase mix (without terminal transferase). Slides were washed, counterstained with DAPI, and mounted with Vectashield.

Microscopy and Imaging

Epifluorescence microscopy was done using an Olympus BX-61 motorized microscope, and confocal microscopy was performed with an Olympus FW- 1000 (Olympus, Center Valley, PA).

Results

Stage-Specific Detection of TOP2B in Mouse Spermatids

Abortive topoisomerase intermediates are known to trigger DDR in somatic cells [27]. Previous results from our group suggested that a type II topoisomerase is involved in the DNA strand breakage observed at mid-spermiogenesis steps. To confirm the nature of the topoisomerase involved, we performed immunofluorescence on Bouin-fixed, paraffin- embedded sections of mouse testes. TOP2B was present in a stage-specific manner throughout spermatogenesis (Figure A1-1, A–C; Supplemental Movie 1 available online at www.biolreprod.org). It was

observed in the chromocenters of Sertoli cells and in the more heavily DAPI-stained and prominent chromatin of spermatogonia. TOP2B was also observed in type B spermatogonia, as well as in some condensed chromatin from preleptotene spermatocytes to stages V–VI pachytene sper- matocytes. It was absent in later pachytene spermatocytes and in round spermatids. However, it was present in nuclear foci of ES and condensing spermatids (CS) between stages IX and I, and weak labeling can be observed in the cytoplasm of CS (from stages I to VI). The flagella were also stained in late elongating and condensing spermatids (stages X to II–III). We also performed immunofluorescence for the detection of TOP2A on testis sections (Figure A1-1 D); spermatogonia and spermatocytes (preleptotenes to secondary spermatocytes) demonstrated strong immunoreactivity, whereas TOP2A was absent from the nuclei of all haploid cells. In ES and CS, only weak cytoplasmic staining was observed.

DSBs in ES

The detection of the active, phosphorylated form of H2AX (H2A histone family, member X; H2AFX), γH2AX, has been associated with the presence of DSBs and is an early signal for induction of the DDR in somatic cells [28–30]. We have previously demonstrated TUNEL positivity of preleptotene to zygotene spermatocytes and ES (stage IX to I), indicating that DNA strand breaks are present in these cells [13]. As the terminal transferase adds modified nucleotides to any free 3'OH DNA termini, TUNEL can not distinguish between single- and double-strand breaks.

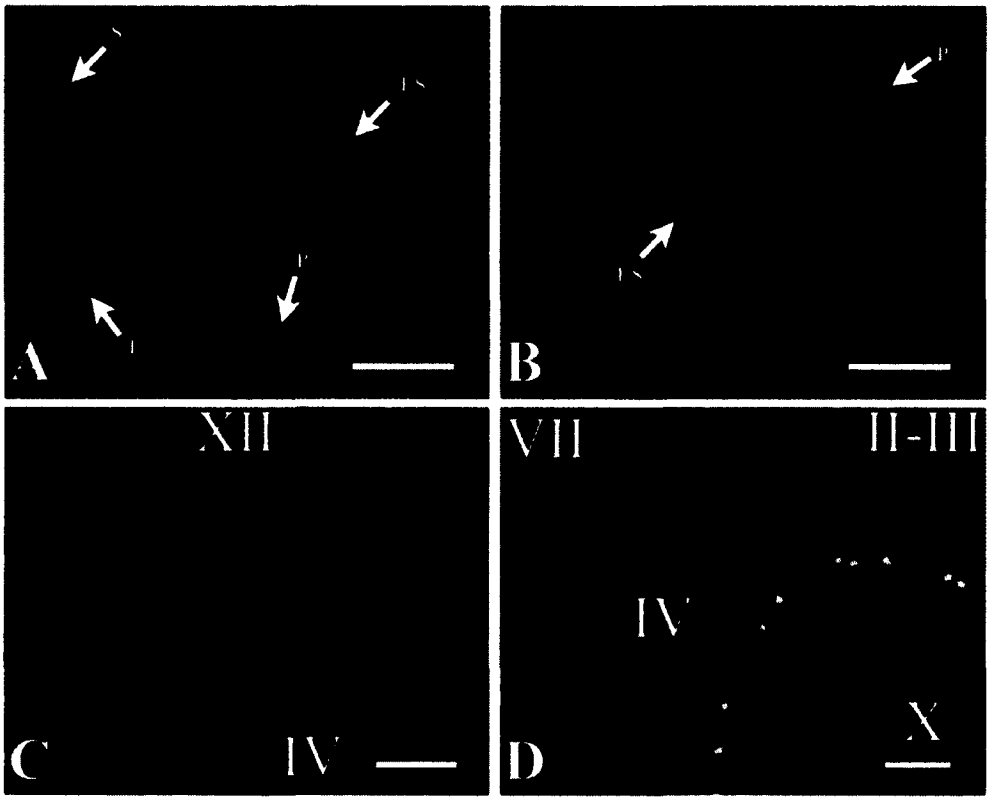

Figure A1-1. Detection of topoisomerase II isoforms on adult mouse testis sections.

A) Overlay of TOP2B immunofluorescence (green) and DAPI nuclear staining (blue) of a stage IX tubule demonstrating the presence of TOP2B in the chromocenters of Sertoli cells (S), heavily DAPI-stained chromatin of leptotene spermatocytes (L), and elongating spermatids (ES). B) Overlay of TOP2B immunofluorescence (FITC; green) and DAPI (blue) nuclear staining of stage X demonstrating TOP2B foci in ES. C) Presence of TOP2B at different stages (IV and XII) D) Overlay of immunofluorescence of TOP2A (green) and TO-PRO3 (red) nuclear staining of stage X demonstrating the nuclear presence of TOP2A in spermatogonia to pachytene spermatocytes (P) but complete absence in spermatids. Bars = 10 μm (A, B) and 20 μm (C, D).

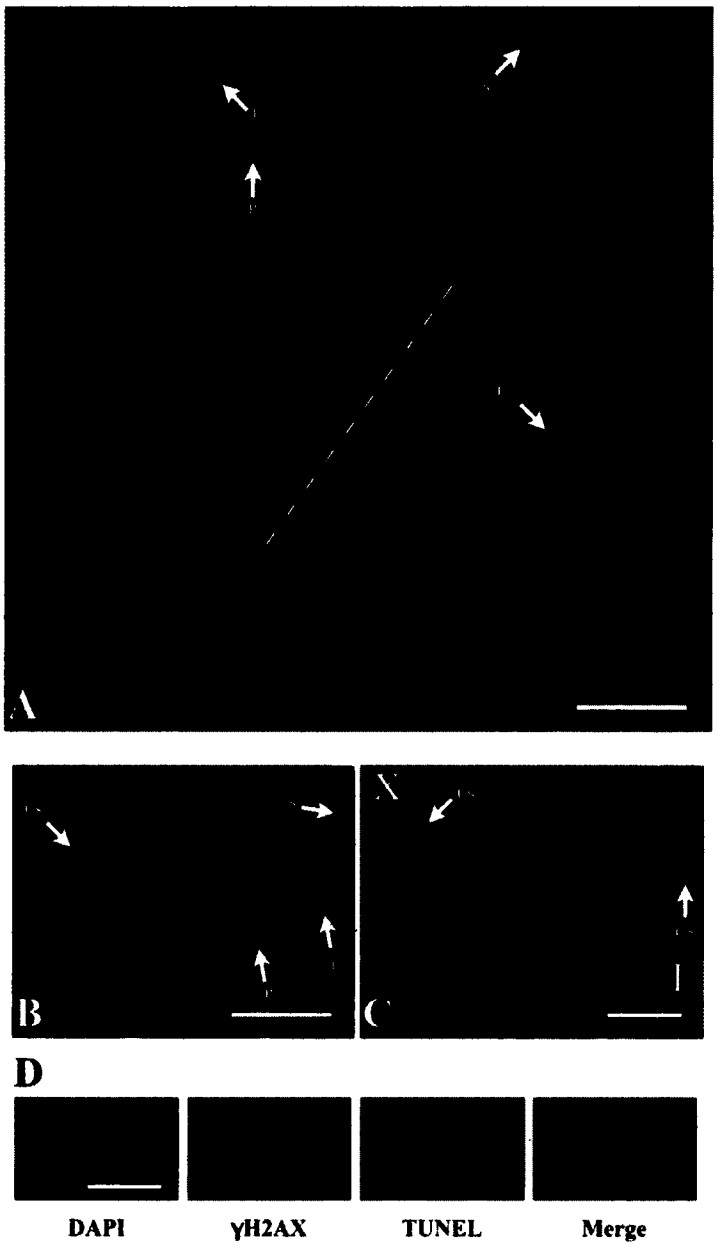

Figure A1-2. Presence of γH2AX in adult mouse testis sections.
Confocal (A-C) and epifluorescence microscopy for the colocalization of TUNEL labeling and γH2AX (D) are shown. A) Immunofluorescence overlay of γH2AX (green) and TO-PRO3 (red) nuclear staining of the end of stage XIII showing the beginning of the activation of H2AX in ES (left side) and the beginning of stage IX (right side), the presence of γH2AX in the sex body of pachythene spermatocytes (P), and nuclear labeling of leptotene spermatocytes (L). S, Sertoli cell. B) Presence of γH2AX at stage X. C) Residual presence of γH2AX at different stages (XI and I). CS, condensing spermatids. D) Colocalization of γH2AX (red) and TUNEL labeling (green) of ES at stage X. Bars=30 μm (A), 20 μm (B, C), and 5 μm (D).

Although previous results from COMET assays indicated that a large proportion of DSBs were present in ES, we seek to establish whether the TUNEL positivity in ES was associated with γH2AX as a reliable biological marker of DSBs. Strong immunoreactivity in cells initiating meiotic recombination (spermatocytes from stages VIII to I) was observed in accordance with our previously reported TUNEL positivity in these cells [13, 24] (Figure A1-2, A-C; see Supplemental Movie 2, available online at www. biolreprod.org, for complete Z-stack of section of Figure A1-2 A; Supplemental Movie 3 available online at www.biolreprod. org). We also show that γH2AX is localized to the sex bodies of pachytene spermatocytes, as this protein is known to play a role in meiotic sexual chromosome inactivation as outlined previously [31, 32]. In haploid cells, γH2AX foci were clearly observed from stages IX to XII. These foci are mainly found in the heterochromatin and rarely in the chromocenter of ES and early CS. In ES, TUNEL labeling coincides perfectly with γH2AX (Figure A1-2 D). It is noteworthy that the presence of a biological marker of double-stranded breakage also indicates that the coincident DSBs seen by TUNEL labeling do not represent an artifact resulting from the tissue preparation that would leave exposed 3'OH ends.

TOP2B, DSBs, and Involvement of TDP1

To check for a possible impairment in TOP2B activity as one potential origin of DNA fragmentation in ES, immunofluorescence was used to detect the presence of the TDP1, an enzyme known to remove topoisomerase I and II adducts [33]. Figure A1-3 displays the localization of TDP1 in the seminiferous epithelium. Pachytene spermatocytes displayed a weak nuclear immunoreactivity for TDP1. At stages IX and X, TDP1 was, however, clearly found in nuclear foci distributed throughout the nucleus of ES and localized to the cytoplasm at subsequent steps. This cytoplasmic localization was found to persist in the residual bodies after spermiation at early stage IX. Because TOP2B is the sole nuclear topoisomerase

detected in the nuclei of ES, the coincidence of TDP1 suggests that aborted TOP2B intermediates are indeed created, leading to DNA strand breakage.

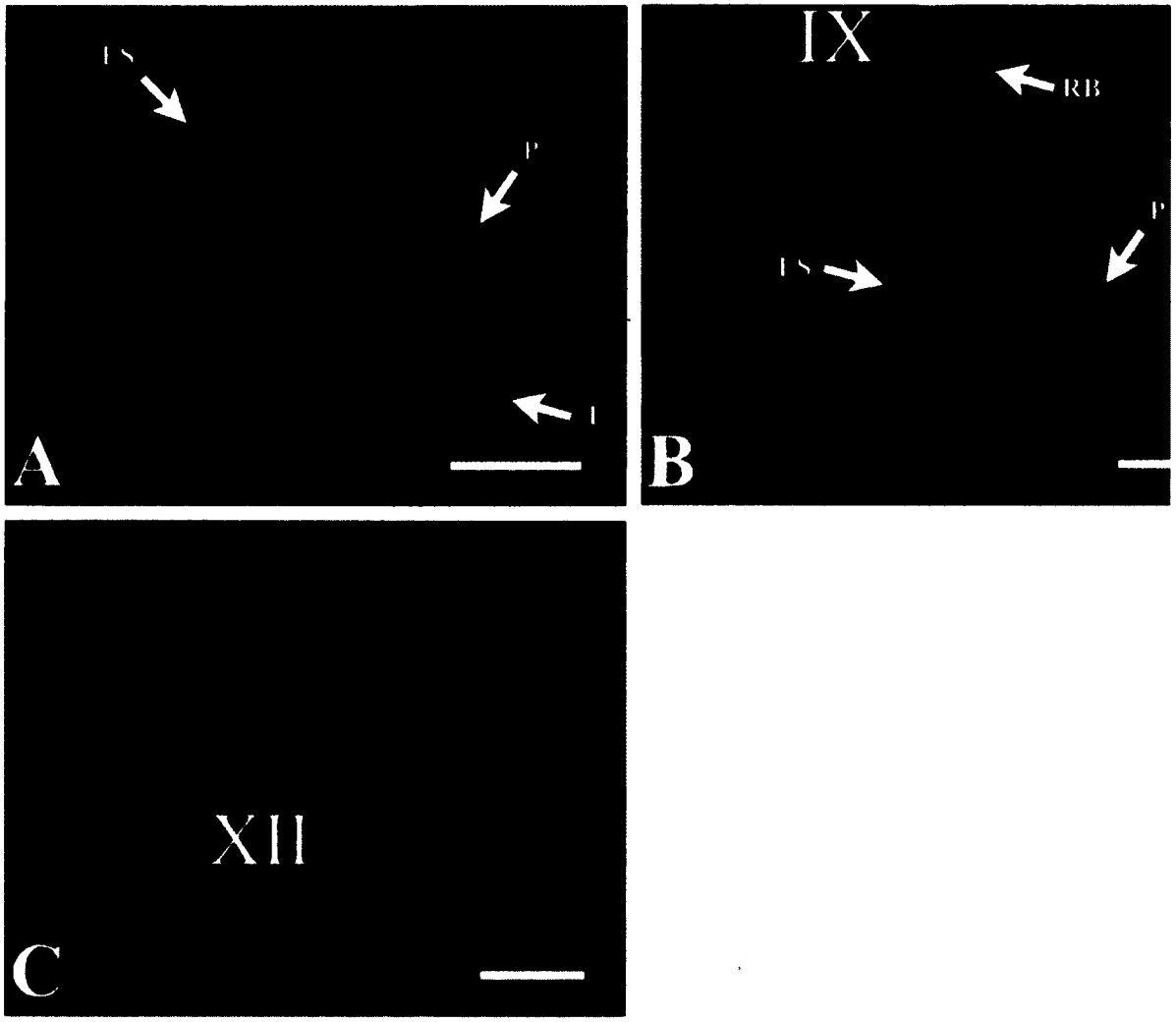

Figure A1-3. Detection of TDP1 on adult mouse testis sections.
A) Overlay of TDP1 immunofluorescence (green) and DAPI (blue) nuclear staining of stage X demonstrating the presence of TDP1 in the nuclei of elongating spermatids (ES). B) Presence of TDP1 in pachytene spermatocytes (P), ES, and residual bodies (RB) at early stage IX. C) Presence of TDP1 at stage XII. Bars = 10 μm (A) and 20 μm (B, C).

Endogenous DNA Polymerase Activity

Because TDP1 dislodges TOP2B cleavable complexes from DNA [34] and H2AX is activated at the site of DSB, we hypothesize that a DNA repair system may be recruited at the site of damage. During the DDR, DNA polymerase activity is required to complete the ultimate repair steps, which are the addition and replacement of intact nucleotides [35], and therefore represents an ideal marker of any active DNA repair process. Using a sensitive in situ endogenous DNA polymerase activity assay on staged squash preparations, a step-specific polymerase activity is shown in the nucleus of ES and CS (steps 9 through 13; Figure A1-4, A and B). Equatorial planes of secondary spermatocytes are also labeled by the endogenous polymerase activity (Figure A1-4 B). Very weak activity is detected in pachytene, leptotene, and zygotene spermatocytes. As shown in Figure 4C, we observed similar results with the addition of terminal transferase. The enzymatic nature of this specific labeling is confirmed by its sensitivity to added EDTA (Figure A1-4 D). DNA polymerase activity in nonreplicating cells can be related only to repair; therefore, these results confirm the presence of an active DNA repair system up to step 14 of spermiogenesis.

From the above, the step-specific detection of the proteins associated with the DSBs is reported on a spermiogenesis map (Figure A1-5). As can be seen, the DDR is concentrated around the chromatin-remodeling steps defined by the detection of hyperacetylated histone H4. In the mouse, these crucial steps (9–13) last about 4 days, after which no expression of DDR factors or repair activity is detected. Hence, most of the genetic integrity of the male gamete is likely determined as spermiogenesis proceeds beyond step 13. Hence, any failure to properly repair the DNA strand breaks within this narrow window of opportunity is expected to lead to persistence of DNA damage in the mature sperm.

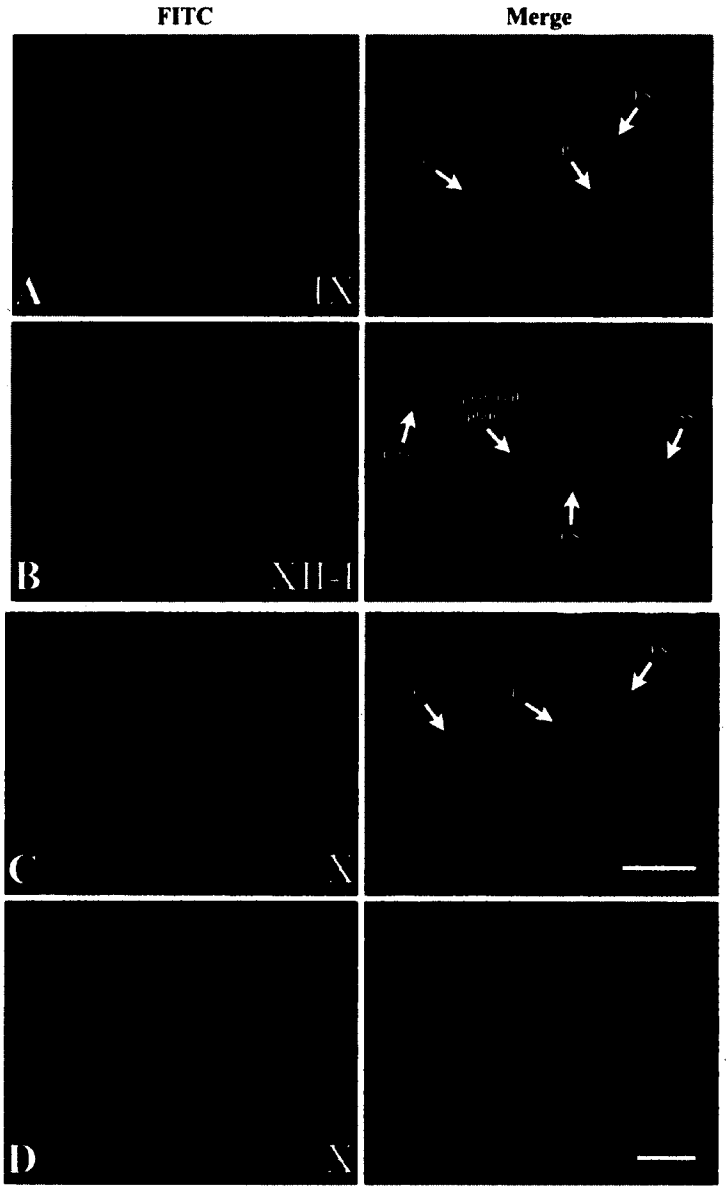

Figure A1-4. *In situ* endogenous DNA polymerase activity assay on squash preparation of staged tubules.

Endogenous DNA polymerase activity during stages IX (A) and XII and I (B). C) TUNEL labeling of stage X. D) Endogenous DNA polymerase activity during stage X in the presence of EDTA. DNA counterstained with DAPI. CdS, condensed spermatid; CS, condensing spermatid; ES, elongating spermatid; L, leptotene spermatocyte; P, pachythene spermatocyte; SS, secondary spermatocyte. Bars = 10 μm (A–C) and 20 μm (D).

Discussion

TOP2B in Spermiogenesis

Spectacular chromatin events take place during spermiogenesis as the somatic chromatin organization is replaced by a more compact, almost crystallized protaminated chromatin [1]. Transient DNA strand breaks may be formed during the process to support the overall change in DNA topology and relieve torsional stress [12]. Through their ability to generate a DSB and catalyze religation, type II topoisomerases appear to be ideal candidates to perform such tasks [14, 22-24].

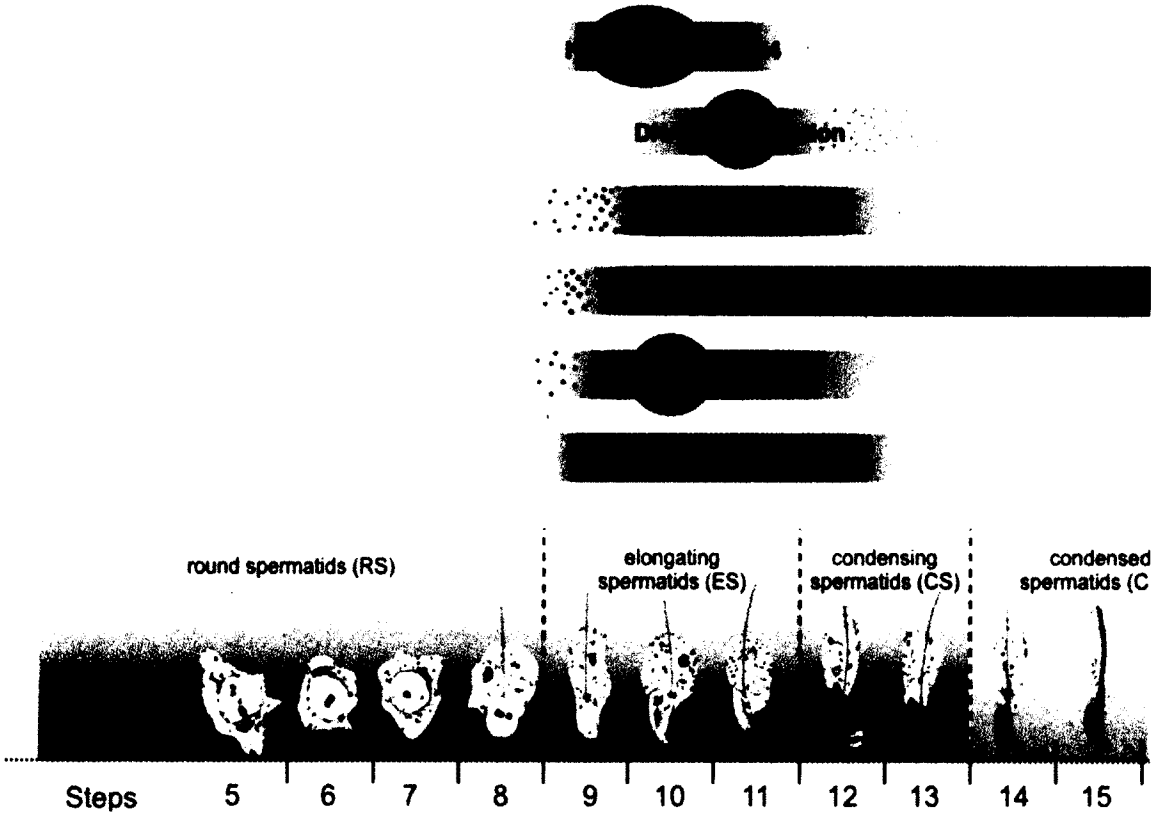

Figure A1-5. Schematic representation of immunofluorescence expression of differents proteins or activity during mouse spermiogenesis.

We have tested anti-topoisomerase II antibodies against TOP2A and TOP2B on seminiferous tubule sections. Only the β isoform was present in foci distributed throughout the nuclei of ES (stages IX to XII), coincident with chromatin remodeling and TUNEL positivity (Figure A1-5) [13]. Shaman et al. [36] found an activable TOP2B in spermatozoa, lending support to the hypothesis that it may assume a role earlier during spermio- genesis.

In the context of the condensing chromatin of ES, the capacity of TOP2B to achieve a complete catalytic cycle is questionable. The coincident phosphorylation of H2AX, a marker of DNA DSBs, supports the possibility that abortive TOP2B intermediates may be generated. Concerns were raised that the strong TUNEL labeling in ES may be a consequence of fixing the cells at a given time point, labeling the DSBs in the process of being religated by the topoII. The γH2AX foci and endogenous polymerase are a clear indication that this is not the case and that a response to endogenous DNA damage is triggered. Although it was shown that γH2AX is not always associated with DSBs, notably in meiotic sexual chromosome inactivation [31, 32] and in monitoring the genomic integrity independent of DNA damage [37], the coincidence of DNA DSBs by TUNEL and by γH2AX foci is striking, strongly suggesting that the activation of this histone variant is triggered by the appearance of DSBs. In addition, the clear detection of DNA polymerase activity indicates that the presence of γH2AX is associated with a genuine DNA damage response. Therefore, we surmise that in ES most of the DNA supercoiling is eliminated flawlessly, but in some cases a cellular signalization of DSB is activated if topoisomerase II activity is hindered. As shown in this paper, this DDR is present in all ES and seems to be part of the normal spermiogenesis program. It is well known that abortive topoisomerase II catalysis triggers a DDR. For instance, collision between topoisomerase adducts and progressing replicative forks or transcription machinery often results in a DNA lesion [38]. In *Saccharomyces cerevisae*, the activity of TDP1 was first related to the hydrolysis of 30-phosphotyrosyl bonds, which are mainly found in topoisomerase I intermediates

[39–41]. However, it was demonstrated recently that the human TDP1 cleaves a broad range of substrates [42] and, in yeast, participates in topoII-mediated DNA damage [33, 34]. Thus, one can hypothesize that TDP1 actively removes TOP2B cleavable complexes during spermiogenesis, leaving a DSB. TDP1 was first identified by mass spectrometry following co-immunoprecipitation of hyperacetylated histone H4 (AcH4) from sonication-resistant spermatid extracts (Joly and Boissonneault, unpublished data). The association of a repair enzyme such as TDP1 with AcH4 is consistent with the known requirement of histone hyperacetylation at damage sites [43]. In this report, the presence of TDP1 in ES was confirmed by immunofluorescence of testis sections. Interestingly, the step-specific appearance of TDP1 during spermiogenesis is coincident with the presence of TOP2B in the nucleus of ES. The presence of TDP1 makes it likely that unprocessed DNA ends from the TOP2B catalysis may be responsible for the formation of DSBs.

DNA Repair System as Part of Normal Spermiogenesis

The presence of γH2AX foci in ES and early CS (stages IX to I) supports previous observations that DNA fragmentation consists of DSBs and provides more insights about this critical step. The presence of γH2AX and endogenous DNA polymerase activity is a clear indication that DDR signaling is taking place during chromatin remodeling in spermatids. Our observation is in agreement with previous work by Meyer-Ficca et al. demonstrating the presence of γH2AX as well as poly(ADP-ribosyl)ation in rat spermiogenesis [44], though, in the latter case, the coincidence with DNA strand breakage was not established. Consistent with these observations, the knockout of the Parp2 gene in mice was associated with severely compromised differentiation of spermatids and delays in elongation [45].

We found endogenous DNA polymerase activity present in ES and CS, whereas much weaker activity was detected in premeiotic and meiotic cells, with

the exception of equatorial planes of secondary spermatocytes. The presence of endogenous DNA polymerase in equatorial planes of secondary spermatocytes may also be related to repair of topoisomerase adducts because the enzyme is required for the segregation of sister chromatids during meiosis I [18]. These results are in accordance with those of Hecht and Parvinen [26], although in their case the labeling was not mainly associated with ES. The presence of DNA polymerase activity up to the CS steps indicates that DNA repair may still proceed to completion despite the major chromatin restructuring in these cells and may explain why DNA strand breaks are normally no longer found in steps 15–16 spermatids even when nuclear decon- densation is performed [13].

Given the haploid character of spermatids, one can assume that they must rely on a process related to nonhomologous end joining (NHEJ) for DNA repair; therefore, it is not surprising that components of NHEJ have been previously associated with spermatids [46–48]. However, processes related to NHEJ are recognized as being error-prone, so spermatids may represent a major source of genetic instability. Unresolved paternal DSBs that have persisted in the mature sperm, leading to high DNA fragmentation level, may not be processed due to the limited repair capacity of the oocyte [49, 50]. In addition, completed NHEJ repair process in these haploid cells may induce mutations that will not be corrected in the zygote and will, therefore, be transmitted to the offspring. Special attention in this regard should be given to the Y chromosome because abnormalities of this chromosome are the second most common cytogenetic anomalies observed in infertile men; micro or partial de novo deletions of the long arm (Yq) have been observed in around 3% of infertile men [51]. The Y chromosome is very vulnerable to DNA deletions because of its inability to participate in recombination repair [52]. Further evidence is needed to establish the consequence of the endogenous DNA strand breaks in fertilization and embryo development.

A Model

Our results are consistent with other evidence from the literature, allowing us to propose what must be considered as an early model of the events taking place during steps 9 to 13 of mouse spermiogenesis (Figure A1-6). The hyperacetylation of the histones H3 and H4, as well as other posttranslational modifications, weakens the affinity of histones to DNA. Somatic nucleosomes are partially removed from DNA, leaving chromatin with free supercoils, which are substrates of TOP2B that relax DNA for the proper deposition of transition proteins and protamines. In this process, part of the TOP2B pool may get trapped in a cleavable complex or may have a partial activity, so TDP1 would be needed. DSBs are left by the cleavage of TDP1 from the DNA-topoisomerase complex, which activates H2AX through the phosphorylation of a member of the phospho-inositide 3-kinase family [28] and triggers the addition by PARP2 of poly(ADP-ribosyl) polymer on surrounding histones [44, 45]. At this point, we hypothesize that elements of the NHEJ are recruited to the DSB. Free DNA ends are then processed, and gaps are filled by a DNA polymerase and ligated. Transition proteins and protamines may be involved in the repair process, as they are known to facilitate DNA ligation both in vitro and in cellulo [53].

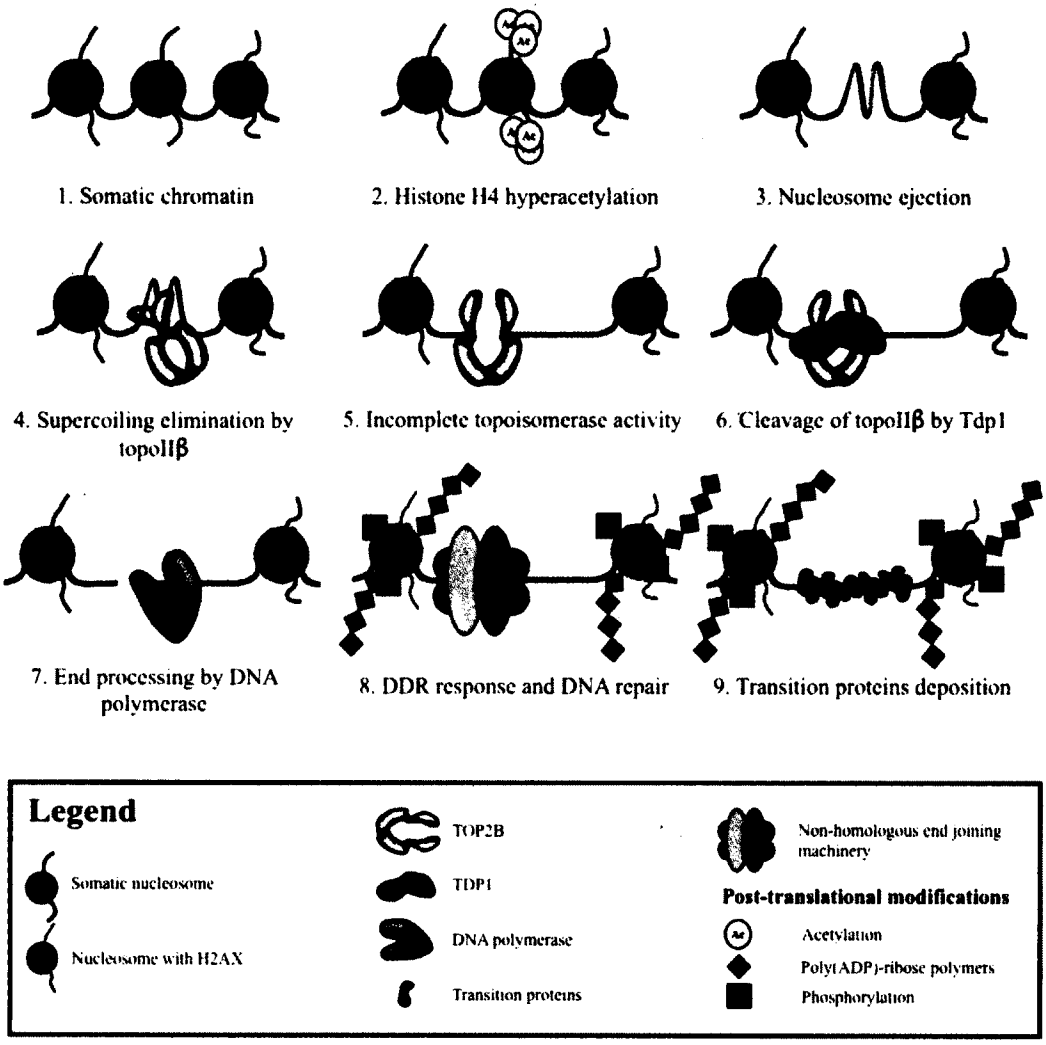

Figure A1-6. Proposed model of DDR and repair induced by abortive topoisomerase II activity during chromatin remodeling steps of mouse spermiogenesis.

Relationship to Human Fertility

The origin of DNA fragmentation in human spermatozoa remains unknown, but multiple sources have been proposed, including abortive apoptosis, abnormal chromatin packaging, and generation of reactive oxygen species [1, 54, 55]. The requirement of a DNA repair system in the normal differentiation program of spermatids supports the concept that transient DNA fragmentation observed during spermatid chromatin remodeling may persist in infertile human

spermatozoa because of impairment in the repair process. Unresolved DSBs resulting from a failure in the rejoining process can have dramatic consequences on the genomic integrity of the developing male gamete. Not surprisingly, increasing evidence from the literature points to alterations in the nuclear integrity of the male gametes as the cause of de novo genetic disorders, developmental and morphological defects, cancer, and miscarriage [56–59]. Furthermore, sperm DNA fragmentation can also compromise assisted reproductive technology (ART) [59]. In conclusion, we provided evidence of a DNA repair system in normal mammalian spermiogenesis likely induced in response to endogenous DNA strand breaks. Given the haploid character of spermatids, an error-prone repair system such as NHEJ must operate. These findings lend strong support to the view that the origin of DNA fragmentation in sperm may lie within the chromatin-remodeling steps in spermatids.

Acknowledgements

The authors wish to thank Dr. Leonid Volkov for his technical advice regarding confocal microscopy and Leila Jaouad for her technical assistance.

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Données supplémentaires

Films disponibles via le site de Biology of Reproduction (www.biolreprod.org):

[Supplemental Movie 1] - "TopoIIbetaz.mpg: Complete confocal Z-stack of a 5 μm testis section. Immunofluorescence of topoisomerase II β (green) and DNA counterstain with TO-PRO3 (red) of two stage X tubules."
http://www.biolreprod.org/content/suppl/2007/11/16/biolreprod.107.064162.DC1/biolreprod.107.064162-1.mpg

[Supplemental Movie 2] - "H2AX1z.mpg: Complete confocal Z-stack of a 5 μm testis section. Immunofluorescence of γH2AX (green) and DNA counterstain with TO-PRO3 (red) at stage IX."
http://www.biolreprod.org/content/suppl/2007/11/16/biolreprod.107.064162.DC1/biolreprod.107.064162-2.mpg

[Supplemental Movie 3] - "H2AX2z.mpg: Complete confocal Z-stack of a 5 μm testis section. Immunofluorescence of γH2AX (green) and DNA counterstain with TO-PRO3 (red) at stage X (up) and stage VI (down)."http://www.biolreprod.org/content/suppl/2007/11/16/biolreprod.107.064162.DC1/biolreprod.107.064162-3.mpg

Épilogue

Suite à cette publication, le laboratoire continue à caractériser les mécanismes impliqués durant la spermiogenèse tant au niveau du remodelage de la chromatine, qu'au niveau de la réparation de l'ADN et des systèmes de réparation disponibles chez les spermatides. La présence d'une réponse aux dommages à l'ADN, principalement observés ici par la phosphorylation du variant d'histone H2AFX, a été par la suite observée chez les spermatides de nombreuses espèces animales et végétales, allant de l'algue *Charas Vulgaris* aux sauterelles (Cabrero, Palomino-Morales, & Camacho, 2007a; Rathke et al., 2010; Wojtczak, Popłońska, & Kwiatkowska, 2008). Au laboratoire, j'ai contribué avec la participation de Geneviève Acteau, étudiante à la maîtrise, à la confirmation de processus similaires chez l'humain (voir l'Annexe 2), le rat et le chien (données non-publiées), démontrant davantage l'aspect de conservation évolutive de ce processus.

Chapitre 2 : Cartographie des cassures d'ADN à l'échelle d'un génome

Genome-wide mapping of DNA strand breaks

Frédéric Leduc, David Faucher, Geneviève Bikond Nkoma, Marie-Chantal Grégoire, Mélina Arguin, Raymund J. Wellinger et Guylain Boissonneault

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Avant-propos

Afin de mieux comprendre les mécanismes responsables du remodelage de la chromatine des spermatides et surtout afin d'évaluer l'impact qu'aurait une réparation inexacte ou incomplète des cassures bicaténaires transitoires engendrés par ceux-ci, nous avons cherché une approche qui pourrait établir la distribution de ces cassures à l'échelle du génome sans perdre la résolution nucléotidique. Or, une telle approche n'existait pas à cette époque. Plusieurs approches mesuraient la fragmentation à l'échelle d'une population de cellules, telle que la migration par électrophorèse en champ pulsé (PGFE), ou à l'échelle d'une cellule par imagerie en utilisant la technique TUNEL ou l'électrophorèse de cellule unique ou COMET, sans pour le moins être quantitatives ou indiquer les endroits précis des cassures. D'autres approches, comme le LM-PCR (ligation-mediated polymérase chain reaction) ou l'extension d'amorce, peuvent déterminer au nucléotide près le site d'une cassure mais sont limitées par l'utilisation d'amorces spécifiques, et donc ne peuvent être appliquées à l'échelle d'un génome.

Il était donc nécessaire de mettre au point une méthodologie qui puisse apporter une réponse à nos interrogations. J'ai entrepris ce projet alors qu'une étudiante à la maîtrise, Marilyne Jolie, avait testé une approche utilisant l'avidine pour capturer des extrémités d'ADN marqué par la terminale transférease (TdT) avec des nucléotides biotinylés. Cette approche souffrait d'un grave problème; il était quasi-impossible d'éluer l'ADN capturé par l'avidine sans utiliser des conditions qui pourraient endommager ou à tout le moins dénaturer l'ADN. Cette problématique a été au cœur de ce projet et s'est avéré critique quelques années plus tard pour la compatibilité avec les technologies de séquençage de 2ème génération (next generation sequencing, NGS) et des génomes comportant un haut taux d'ADN répété (i.e. celui de la souris et de l'humain). C'est pour cette raison que mes premières tentatives utilisaient la PCR comme moyen d'enrichir les séquences endommagées.

Cette approche, appelée TAM-PCR (terminal transferase and adaptor-mediated PCR), était inspirée de la méthode TD-PCR (terminal transférase-dependent-PCR) mais se différenciait par la ligation supplémentaire d'un duplex d'ADN comme amorce universelle sur l'extrémité non-endommagée (voir Figure AV1). Bien que je puisse enrichir des séquences spécifiques d'un modèle plasmidique avec cette approche, j'obtenais de nombreuses séquences non-spécifiques probablement provenant de l'utilisation d'une amorce oligo-dT aussi peu spécifique et demandant une basse température d'hybridation (40-43°C) pendant le PCR d'enrichissement. De plus, cette approche comportait un problème majeur; plusieurs génomes possèdent de longues séquences d'adénine qui donneraient constamment de faux positifs.

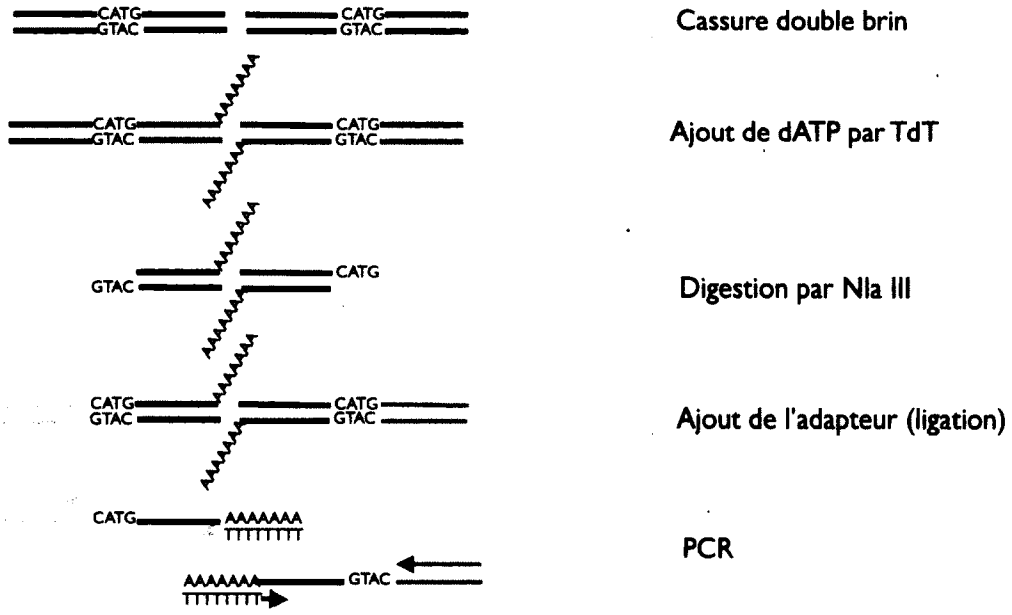

Figure AV2-1. Schéma explicatif de l'approche TAM-PCR (terminal transferase and adaptor-mediated PCR).

Cette approche fut abandonnée et nous sommes revenus à l'approche utilisant la capture avidine-biotine. Pour ce faire, j'ai mis au point une façon de contourner l'obstacle d'élution en effectuant la capture et l'amplification dans un seul et même tube. Cette autre méthode, appelée *damaged DNA immunoprecipitation* ou dDIP, est décrite en détails dans la section Prologue du présent chapitre. Étant à la base de

l'approche finale décrite dans l'article du présent chapitre, je trouvais important de la décrire en détails. La méthode TAM-PCR a de plus été mise de côté à cause de son incompatibilité avec le séquençage de 2ème génération et sa capacité de capture inflexible, et non par manque de spécificité.

J'ai conçu les méthodologies et les expériences, a effectué la majorité des expérimentations, a analysé et compilé les résultats et a rédigé l'article scientifique. David Faucher, étudiant au doctorat au laboratoire de Professeur Raymund Wellinger, a fourni les souches de levures, fait la culture et l'induction de ces souches, et a effectué certaines expériences impliquant la technique de buvardage de type Southern. Geneviève Bikond Nkoma, Marie-Chantal Grégoire et Mélina Arguin ont participé à la mise au point de certaines étapes de la technique dDIP. Professeur Raymund Wellinger a contribué à la réflexion scientifique et à la révision du manuscrit. Professeur Guylain Boissonneault a guidé la démarche expérimentale proposée par Frédéric Leduc et a contribué à la rédaction de l'article scientifique.

Prologue

Pour mettre au point une approche permettant d'enrichir les régions génomiques endommagées des spermatides en processus de remodelage de la chromatine, j'ai décidé d'utiliser un modèle plasmidique, soit le plasmide pcDNA3, pour tester les différents paramètres à optimiser. Il était donc facile de simuler une cassure bicaténaire à l'aide d'une enzyme de restriction et de suivre par PCR les séquences flanquantes grâce à des paires d'amorces spécifiques. Dans la Figure P2-1, on retrouve la stratégie alors envisagée pour établir la cartographie génomique des régions endommagées des spermatides allongeantes. Brièvement, l'ADN génomique était marqué par la TdT et des nucléotides biotinylés, fragmenté par une endonucléase à coupure fréquente (NlaIII) et capturé par un support recouvert de streptavidine (tubes, plaque ELISA, billes magnétiques). L'ADN capturé était alors amplifié de façon non-spécifique par DOP-PCR (*degenerate oligonucleotide primed PCR*) et marqué pour en faire des sondes pour l'identification sur biopuces ou cloné puis séquencé par la méthode Sanger.

À cette époque, le séquençage de deuxième génération commençait à être commercialisé (par Roche) et était encore très expérimental et dispendieux; la technique de choix était la technologie des biopuces dont les capacités et les couvertures étaient en constante amélioration. De plus, les tubes recouverts de streptavidine, alors utilisés pour la génération d'ADN complémentaire à partir d'ARN, offraient un excellent compromis avec une capture et l'amplification PCR dans un seul et même tube, contournant le problème épineux de l'élution de l'ADN de la streptavidine. Nous avons d'abord testé la capture de fragment d'ADN par ces tubes en utilisant un produit de PCR de 1 010 pb (provenant de l'amplification du plasmide pcDNA3) où nous avions introduit lors du PCR des nucléotides biotinylés (voir Figure P2-2). Nous avons ainsi optimisé les conditions d'incubation et de lavage pour assurer une capture spécifique.

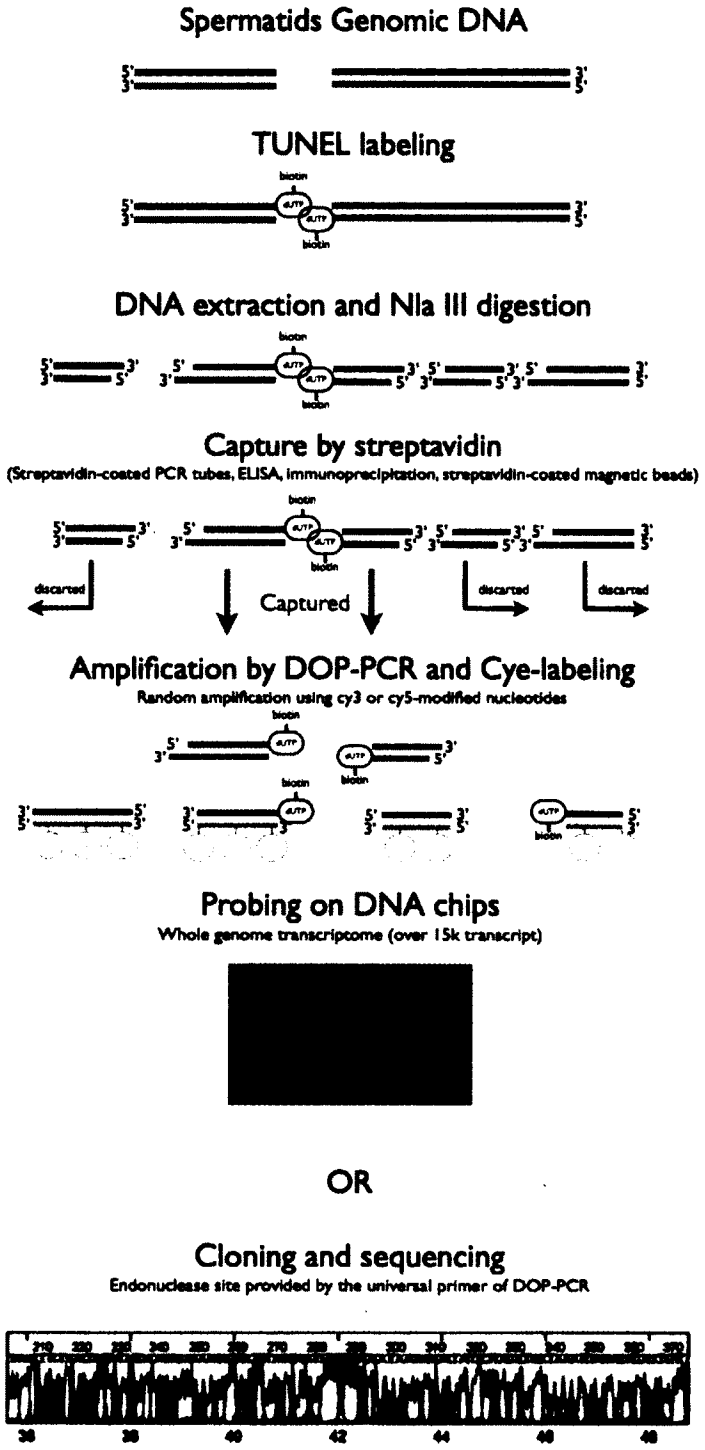

Figure P2-1. Application de l'approche avec les tubes recouverts de streptavidine pour la découverte de régions endommagées durant le remodelage de la chromatine des spermatides

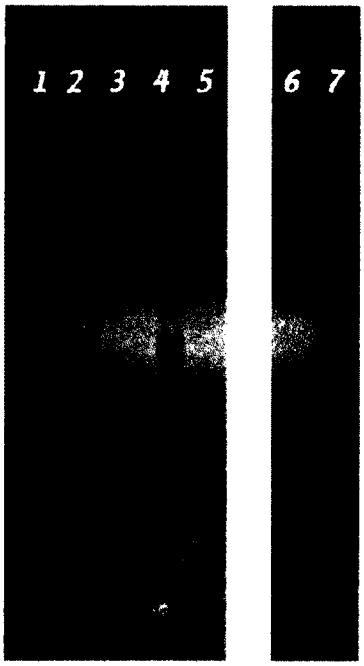

Figure P2-2. Capture et amplification d'un produit de PCR de 1010 pb utilisant des tubes recouverts de streptavidine.
Ligne 1; 1 kb plus DNA ladder (Invitrogen). Lignes 2 et 4; produits de PCR avec l'incorporation de dUTP-Biotine. Lignes 3 et 5; produits de PCR sans dUTP-Biotine. Ligne 6; témoin négatif de PCR. Ligne 7; témoin positif PCR (5 pg du plasmide pcDNA3)

Par la suite, nous avons testé la capture avec les tubes recouverts de streptavidine avec un modèle plasmidique qui se rapprochait davantage à la méthodologie proposée pour l'enrichissement des séquences endommagées chez les spermatides allongeantes. Cette stratégie, décrite à la Figure P2-3, permettait de mettre en évidence la capture spécifique de deux fragments, α et β, marqués par la TdT par rapport à l'enrichissement d'un fragment non-marqué (témoin négatif), le fragment ε. Il était donc possible de vérifier la présence de ces fragments par une amplification PCR avec les amorces spécifiques visant chaque fragment et une visualisation sur gel d'agarose.

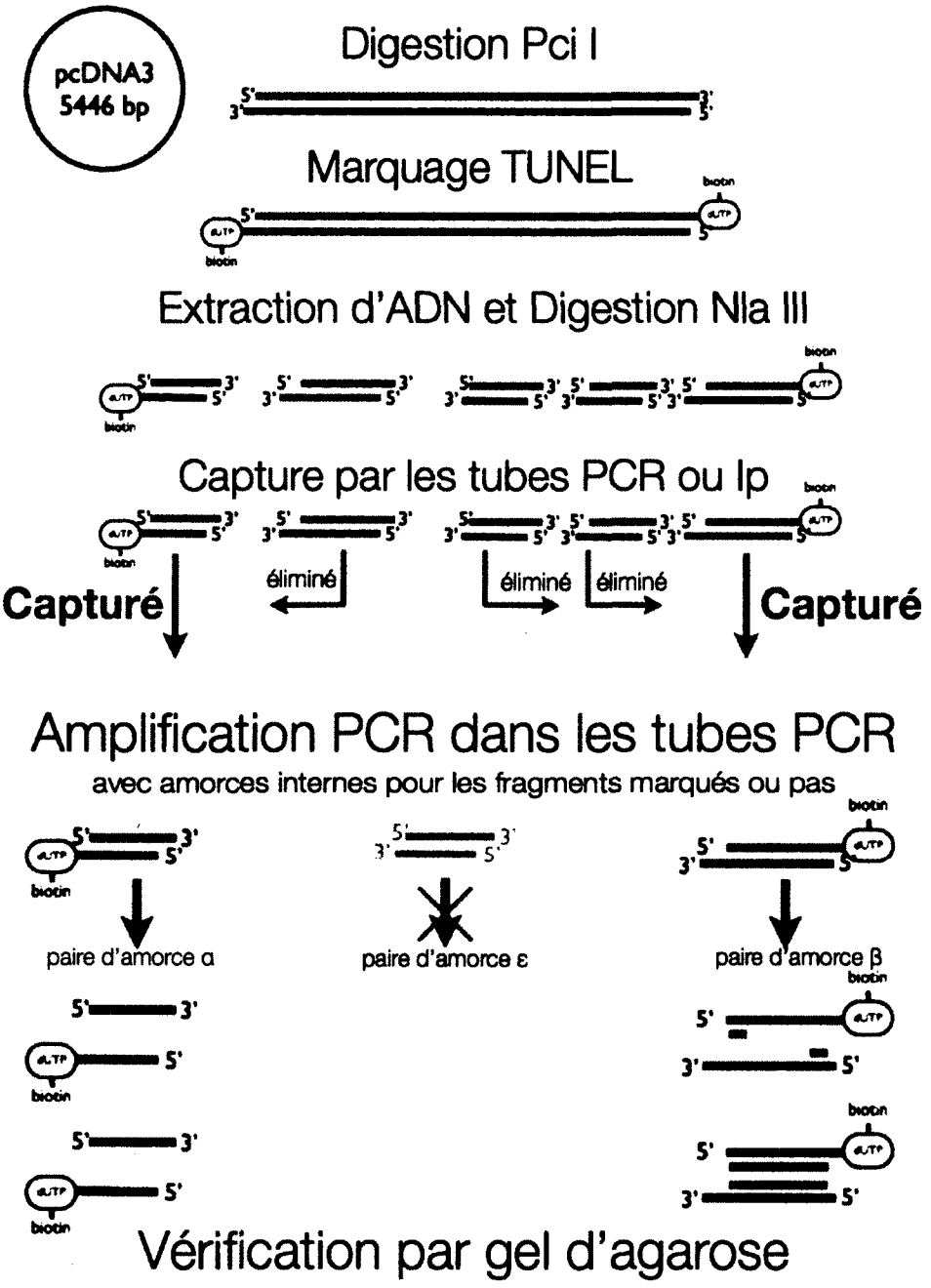

Figure P2-3. Stratégie utilisée pour vérifier la spécificité de la capture des séquences d'ADN endommagées.

Tel que démontré dans la Figure P2-4, la capture spécifique des fragments α et β peut être effectué par cette approche avec une grande spécificité, car il y a absence de capture et d'amplification du fragment ε. Par contre, les conditions d'amplification PCR n'était pas tout à fait au point (grande présence de dimère d'amorce, amplification non-

spécifique pour l'amplicon α), alors nous avons opté pour une amplification PCR multiplexe qui nous permettait de visualiser la capture des trois amplicons dans un seul tube. Cette approche a été utilisée par la suite pour l'optimisation de l'immunoprécipitaiton (voir Figure P2-5 et Figure A2-2).

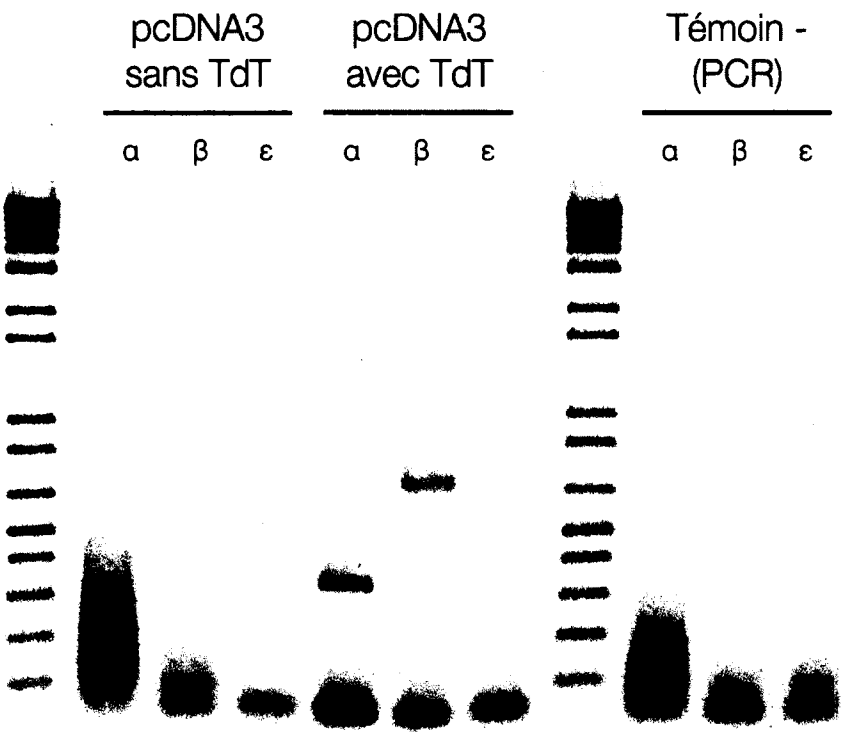

Figure P2-4. Amplification PCR des séquences endommagées, marquées par la TdT et capturées dans des tubes recouverts de streptavidine.

Bien que la capture avec les tubes recouverts de streptavidine offrait de nombreux avantages, ses limitations devenaient de plus en plus apparentes; les tubes avait une capacité limitée de capture et celle-ci était aussi en fonction du volume (surface) appliqué. Comme il était impossible de prédire la quantité exacte de cassures dans nos échantillons d'ADN, cette limitation posait un réel problème. Pour contourner une fois de plus l'élution problématique de la biotine captée par l'avidine, nous avons choisi de capturer les fragments marqués grâce à un anticorps anti-biotine qui permettrait une élution plus facile mais une capture tout aussi spécifique. Comme présenté à la Figure P2-5, nous avons couplé les anticorps anti-biotine à des billes magnétiques recouvertes de protéine G pour créer un immunocomplexe efficace et

spécifique, ainsi que deux types de nucléotides biotinylés; le *damaged DNA immunoprecipitation* ou dDIP était né. Dans l'article présent dans ce chapitre, nous avons testé l'efficacité et la spécificité de cette nouvelle approche avec un modèle plasmidique et le modèle de la levure *S. cerevisiae* pour un contexte de cassure *in vivo*.

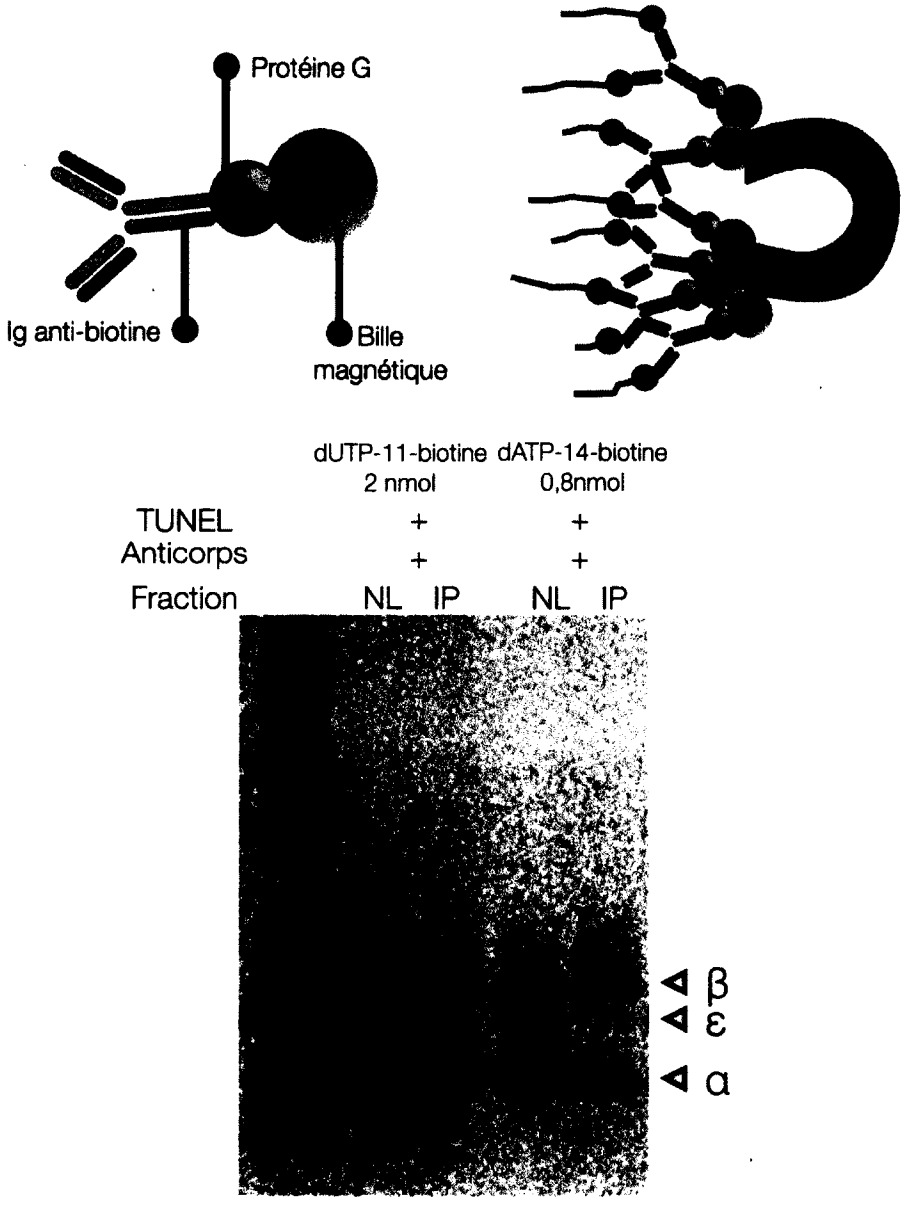

Figure P2-5. Résultats de PCR multiplexe avec trois paires d'amorces de la capture par immunoprécipitation des séquences endommagées du plasmide pcDNA3, marquées à l'aide de deux nucléotides biotinylés différents.

NL, fraction non-liée; IP, fraction immunoprécipitée.

Résumé

La détermination des dommages à l'ADN cellulaire a été jusqu'à maintenant limitée à une évaluation globale de l'intégrité génomique alors que les cartographies à la résolution nucléotidique ont été circonscrites à des régions précises par l'utilisation d'amorces spécifiques. Par conséquent, seules quelques séquences d'ADN peuvent être étudiés et de nouvelles régions d'instabilité génomique peuvent difficilement être découvertes. En utilisant le modèle bien caractérisé de la levure, nous proposons une stratégie simple de cartographie des cassures d'ADN à l'échelle d'un génome sans compromettre la résolution nucléotidique. Cette technique, appelée "damaged DNA immunoprecipitation" (dDIP), s'appuie sur l'immunoprécipitation et la technique "terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling" (TUNEL) pour capturer l'ADN aux sites de cassures. En combinaison avec les technologies de biopuces ou de séquençage de deuxième génération, la dDIP permettra aux chercheurs de cartographier à l'échelle d'un génome les cassures d'ADN, ainsi que d'autres types de dommages à l'ADN, et établir un profile de gènes et/ou de séquences intergéniques altérés dans différentes conditions expérimentales. Cette méthode de cartographie pourrait s'appliquer à plusieurs domaines de recherche tels que le vieillissement, la découverte de nouveaux composés génotoxiques, le cancer, la méiose, les dommages à l'ADN causés par les radiations et par l'oxydation.

Abstract

Determination of cellular DNA damage has so far been limited to global assessment of genome integrity whereas nucleotide-level mapping has been restricted to specific loci by the use of specific primers. Therefore, only limited DNA sequences can be studied and novel regions of genomic instability can hardly be discovered. Using a well-characterized yeast model, we describe a straightforward strategy to map genome-wide DNA strand breaks without compromising nucleotide-level resolution. This technique, termed "damaged DNA immunoprecipitation" (dDIP), uses immunoprecipitation and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling (TUNEL) to capture DNA at break sites. When used in combination with microarray or next-generation sequencing technologies, dDIP will allow researchers to map genome-wide DNA strand breaks as well as other types of DNA damage and to establish a clear profiling of altered genes and/or intergenic sequences in various experimental conditions. This mapping technique could find several applications for instance in the study of aging, genotoxic drug screening, cancer, meiosis, radiation and oxidative DNA damage.

Introduction

Currently available methods to assess DNA damage include electrophoretic techniques such as pulse-field gel electrophoresis (PFGE)[1] or single-cell electrophoresis (Comet assay)[2] for a global assessment of DNA fragmentation. Ligation-mediated polymerase chain reaction (LM-PCR) is also commonly used for quantitatively displaying DNA lesions in mammalian cells because it combines nucleotide-level resolution with the sensitivity of PCR[3] but is limited by the use of sequence-specific primers. All of the aforementioned approaches suffer from one important limitation as they they do not allow mapping of DNA strand breaks on a genome- wide scale and cannot identify new sensitive sites or hotspots harboring such break sites. Therefore, a reproducible method for the genome-wide mapping of DNA

strand breaks would be useful to study their global distribution all at once and monitor any alteration in damage profile under different experimental conditions. Here, we provide a detailed description of a straightforward strategy, termed "damaged DNA immunoprecipitation" or "dDIP". This method uses the immunoprecipitation of biotin-modified nucleotides added by the terminal deoxynucleotidyl transferase (TdT) at sites of DNA damage (see Figure A2-1). Although a similar approach has been used recently to map nuclear receptor-dependant tumor translocations [4], we describe for the first time its genome-wide application resulting from the development and optimization of this method by our group over the past three years. Because of its potential widespread use in genome research, we provide the important experimental details and key findings for the reliable capture and enrichment of damaged DNA sequences in the form of strand breaks.

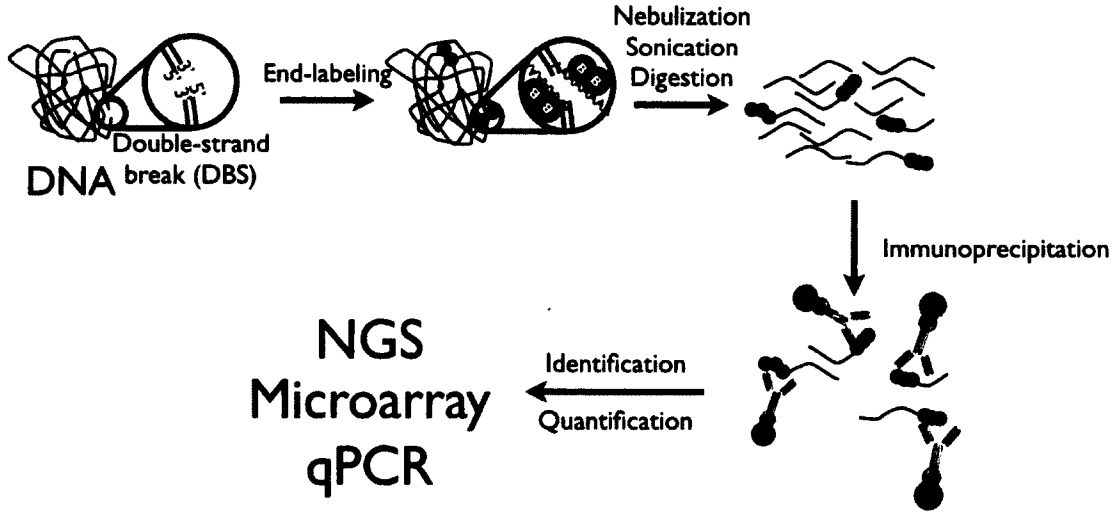

Figure A2-1. Protocol for damaged DNA immunoprecipitation.
DNA breaks are end-labeled by the incorporation of biotin-dNTP at 3'OH termini by the terminal transferase. The labeled DNA is further digested in fragments of suitable size for immunoprecipitation. DNA fragments are immunoprecipitated with anti-biotin antibodies and protein G-coated magnetic beads. Enriched DNA fragments can be identified and quantified using qPCR, microarrays or next-generation sequencing (NGS).

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Plasmid preparation and end-labeling

To demonstrate the specific capture and sensitivity of DNA strand breaks in vitro, we used a plasmid model (pcDNA3, Invitrogen, Burlington, ON, Canada) (Figure 2). First, pcDNA3-transformed DH5α *E. coli* were grown overnight and the plasmid was purified using a Qiagen Plasmid Midi Kit (Qiagen Inc., Mississauga, ON, Canada). Plasmid integrity was checked by 0.8% agarose gel electrophoresis (Promega corp., Madison, WI). Yield and purity was determined by spectrometry (Ultrospec 2100 pro, GE Healthcare, Piscataway, NJ). A unique double-stranded break was created using the endonuclease PciI (New England Biolabs, Ipswich, MA) by digesting one mg of plasmid with 10 U of enzyme in a final volume of 20µl for 1h at 37°C in the recommended buffer (NEB3). Thermal inactivation of PciI was obtained by raising the reaction temperature to 80°C for 20 min. This preparation is referred to as "digested" (Dig). Undigested pcDNA3 plasmid was used as negative control and termed "N".

The plasmid DNA was then labeled by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling (TUNEL) using a recombinant terminal transferase (Roche Applied Science, Laval, QC, Canada) and according to the standard tailing protocol from the supplier. Briefly, 4 mg of linearized (Dig) and undigested (N) pcDNA3 plasmids were incubated in TUNEL reaction mix containing 5 mM $CoCl_2$, 0.20 mM of biotin-16-dUTP, 0.5 mM dATP and 1600 U of TdT (Roche Applied Science) at 37°C for 1 h in a final volume of 80 µl. Negative control was obtained using PciI-

digested pcDNA3 plasmids by omitting the TdT (referred as Dig-) in the reaction. Unmodified dATP was added to the labeling mix to increase the number of biotin-modified nucleotides at 3'OH DNA termini. TdT prefers dATP as substrate and can add up to 5 times more dATP than other nucleotides at identical concentration. Hence, we have included dATP to the labeling mix to increase the length of the end-labeling and consequently the number of biotin- modified nucleotides added to the termini of each DNA breaks. This drastically increases the efficiency of immunoprecipitation with a more complex genomic background, such as *E. coli* and *S. cerevisiae.* Quality of the end-labeling was demonstrated by poly (A) tailing using unmodified dATP. The end-labeling reaction was arrested by the addition of 8 µl of 200 mM EDTA, pH 8. To remove TdT, samples were precipitated by adding 8 µl of 5 M LiCl and 300 µl of cold absolute ethanol and kept at -20°C for 2 hours. The DNA was pelleted by centrifugation at 4°C at 13,000 rpm for 15 minutes by an IEC Micromax countertop centrifuge. The supernatant was then discarded and replaced by 200 µl of ice-cold 70% ethanol. After a second centrifugation of 10 minutes at 4°C, the supernatant was discarded and the DNA pellets were air-dried for 10 minutes. DNA precipitation was preferred to phenol-chloroform extraction or to commercially available DNA affinity columns since phenol/chloroform extrac- tion will partition biotinylated DNA to the organic layer whereas affinity columns are usually unfit for very large DNA fragments or single-stranded DNA (due to end-labeling) which will result in important loss during purification.

Plasmid DNA fragmentation

In order to obtain fragments of suitable sizes for immunopre- cipitation, DNA samples were digested to fragments ranging from 4 to 720 bp by the endonuclease NlaIII in NEB4 buffer supplemented with 100 µg/ml of bovine serum albumin (BSA) for 1–3 h at 37°C, followed by the thermal inactivation at 65°C for 20 min. Labeled DNA fragments were stored at -20°C until immunoprecipitation. We first immunoprecipitated from 6 fmol to 0.6 amol of plasmid (representing about 33,000 to

<10 pcDNA3 copies), and finally by real-time PCR quantification (qPCR), 1 µg, 0.1 µg and 0.01 µg (0.278 pmol to 2.78 fmol) of DNA were immunoprecipitated in duplicate.

Yeast strain

DFY046. This yeast strain DFY046 (**Mat a** *leu2-3,112 trp1-1 ura3-1 can1-100 ade2-1 his3-11,15 RAD5 bar1DELTA::KMX*) is derived from S288c background in which the BAR1 gene encoding a yeast protease was replaced by KMX using a classical PCR deletion technique [5]. The HO cleavage site 5'-AGTTTCAGCTTTCCGCAACAGTATAATTTTATAAA-3' was integrated 45 nt downstream of the start codon of the *PHO5* gene using the Splicing by Overlapping Extension PCR (SOE-PCR) technique [6]. The resulting DNA fragment containing an HO cleavage site was cloned into XhoI-XbaI (New England Biolabs) restriction sites of the YIp-PHO5 plasmid (obtained from Dr Luc Gaudreau, Université de Sherbrooke). The resulting YIp-PHO5-HO plasmid was linearized with SalI (New England Biolabs) and integrated into the *PHO5* genomic locus of the *bar1D::HIS3* strain. Finally, cells having lost the plasmid backbone containing the *URA3* cassette were selected by growth on 5-FOA media. All integration and pop-out steps were confirmed by PCR and Southern blot analyses. The plasmid YcpHOcut4 [7] was transformed into this DFY046 strain.

Growth and HO endonuclease induction

Yeast cells were grown overnight in Yc-Ura media containing 2% glycerol and 2% lactic acid, pH 6. Cultures were diluted to 1 x 10^6 cells/ml in 100 ml of Yc-Ura + raffinose and regrown until they reach exponential phase (<1 x 10^7 cells/ml). Cells were then arrested in G1 phase of the cell cycle by the addition of 0.5 µg/ml α-mating factor (Bioshop Canada inc., Burlington, ON, Canada) and incubated for 3 hours. Cultures were split into 50 ml aliquots and glucose (2% final concentration) was added to the control uninduced sample, referred to as N, and galactose (2% final concentration) to the experimental culture, referred to as I (induced). Cells were

incubated for 90 min before extracting DNA using Qiagen genomic-tips 100/G kit (Qiagen Inc.). Genomic DNA concentration was first determined by spectrometry and then verified by electrophoresis on 0.6% agarose gel.

Yeast DNA end-labeling

To demonstrate the specific capture of DNA strand breaks induced in vivo, yeast DNA was end-labeled by TUNEL as described above with modifications. Each end-labeling reaction was carried out using the same ratio of DNA/labeling mix and was scaled up depending on the experiment. Thus, 0.5 µg of genomic DNA was incubated in TUNEL reaction mix at 37°C for 40 min in a final volume of 20 µl of TdT reaction buffer, 5 mM of $CoCl_2$, 0.10 mM of biotin-14-dATP (Invitrogen), 0.25 mM of dATP and 200 U of TdT. As a negative control for DFY046 DNA end-labeling, DNA was incubated without TdT in the reaction mix and referred to as I- or N-, when using HO-induced and non-induced DNA respectively. After end-labeling, samples were precipitated as described above and dissolved in NEB4 buffer supplemented with BSA for Bsp1286I digestion (New England Biolabs). Restriction digestion was done at 37°C for 3 h followed by thermal inactivation at 65°C for 20 min. Immunoprecipitations of 0.5 µg of genomic DNA were done in triplicate with the exception of the telomere immunoprecipation experiment.

Immunocomplex and immunoprecipitation

For immunoprecipitation of labeled DNA, goat anti-biotin antibody (Abcam inc., Cambridge, MA) coupled to protein G- coated magnetic beads (Dynabeads Protein G, Invitrogen) were used as immunocomplex. We used a ratio of 0.5–1 µg of labeled DNA per 50 µl of immunocomplex (original volume of beads) and upscaled this DNA/immunocomplex ratio when needed. The necessary volume of beads was pooled and washed three times with 1 ml of 0.1 M sodium citrate buffer, pH 5. The beads were then incubated for 3–5 hours at room temperature using a Mini Labroller rotator (Labnet International Inc., Edison, NJ), in citrate buffer containing 1.5 µg of goat anti-

biotin antibody per 50 µl of original beads volume. The beads were then washed three times with 1 ml of citrate buffer, and resuspended in 150 µl of citrate buffer for each immunoprecipitation. One hundred and fifty µl of the immunoprecipitation complex (beads + antibody) was added in a sterile 0.2 ml PCR tube. The tubes were placed on a magnetic block (MPCH-9600, Dynal, Oslo, Norway) for magnetic capture and the supernatant discarded. Labeled DNA samples were diluted in citrate buffer in a final volume of 50 µl, added to the beads and vortexed slowly. To determine immunoprecipitation efficiency, one immunoprecipitation reaction was kept at -20°C until qPCR quantification to be used as reference DNA before IP (DNA_{input}). Labeled DNA was incubated overnight at 4°C with the immunocomplex using a rotating wheel. Samples were then placed on the magnetic block for 2 min, and the supernatant withdrawn. The pellets were washed once with 150 µl of a low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), then with a high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl) followed by a third washing step in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8). Finally the pellet was washed twice with TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8). To elute the captured DNA, beads were incubated at 90°C for 20 minutes in 150–200 µl of pre-heated TE buffer (10 mM tris, pH 8, 1 mM EDTA) in a dry bath (AccuBlock, Labnet International, Inc., Woodbridge, NJ) with frequent stirring. After the incubation, samples were quickly put on the magnetic block, and the supernatant was transferred to a sterile 1.5 ml eppendorf (referred to as DNA_{IP}) and was kept at -20°C until analysis.

Immunoprecipitation of yeast telomeres

To match the detection levels observed by southern blots, we have immunoprecipitated 5 µg of uninduced yeast DNA previ- ously digested with a ratio of 10 U per mg of DNA by XhoI and PstI (New England Biolabs). These endonucleases have cutting sites within the conserved telomere proximal Y' repeat element releasing a <1.2 kb and <1.0 kb terminal restriction fragment (TRF) respectively, which includes

the terminal 0.35 kb TG1-3 repeats [8]. The dDIP was carried out as outlined above with the following modifications. The end-labeling reactions were upscaled and 1.5 ml eppendorf tubes were used to accommodate larger volumes. The DynaMag^{tm}2 (Invitrogen) capture block was then used to isolate the magnetic beads. The final elution was carried out in 200 µl of TE buffer so as to concentrate the immunoprecipitated DNA.

Immunoprecipitation of labeled plasmid DNA within an *E. coli* genomic context in agarose plugs

We have adapted a rapid bacterial pulsed-field gel electrophoresis (PFGE) protocol [9] to immobilize DH5α *E. coli* and isolate DNA content with minimal DNA damage due to handling. Fifty ml of overnight-cultured DH5α *E. coli* cells transformed with the pcDNA3 plasmid were embedded in 1% low melting agarose in TE buffer by mixing an equal volume of bacterial culture and 2% low melting agarose in TE (maintained at 37°C after melting) and transferred in 100 µl-plug cast. Once solidified at room temperature or 4°C for 10 to 15 min, plugs were removed from the mold and transferred in 10 ml of lysis buffer (50 mM Tris, 50 mM EDTA, 1% N-lauryl-sarcosine, 0.1 mg/ml proteinase K, pH 8) in a 125 ml Nalgene conical flask, and incubated for 2 h in a shaking water bath (230 rpm at 54°C). Plugs were washed thrice at 54°C for 10 minutes with 5–10 ml of water in a shaking water bath and the same washing steps were repeated this time using TE buffer. Three plugs were transferred into 2 ml eppendorf tubes and pre-incubated in 300 µl of NEB3 (ratio of 100 µl per plug, New England Biolabs) supplemented with 10 µg/ml of Rnase A for 30 min at 37°C in a water bath. The reaction buffer was withdrawn, replaced by fresh buffer containing 30 U of PciI per plug and the tubes were incubated for 4 h at 37°C in a water bath. Plasmid digestion was confirmed by agarose gel electrophoresis (data not shown).

The plugs were washed three times with 1 ml of TE, and pre-incubated in 300 µl (100 µl per plug) of TUNEL mix (1X TdT reaction buffer, 5 mM of $CoCl_2$) for 30 min at

room temperature. The TUNEL mix was then removed and replaced with a fresh mix containing 2,400 U of TdT (800 U per plug) and 6 nmol of biotin- 16-dUTP (2 nmol per plug) and the eppendorf tubes were incubated overnight at 4°C on a rotating wheel. The tubes were placed in a water bath for 3 h at 37°C. Then, plugs were washed three times with 1 ml of TE and were digested with 1.5 U of β-agarase I according to the manufacturer (New England Biolabs). DNA was precipitated in 100% ethanol overnight at -20°C with the addition of 1 µl of glycogen (Roche Applied Sciences) as carrier, centrifuged at 13 k rpm at 4°C for 20 min, washed with 70% ethanol, air-dried for 15 min and resuspended in 30 µl of NEB4 buffer supplemented with 100 µg/ml of BSA and 30 U of Nla III. The DNA was digested for 1 h at 37°C, and thermal inactivation was allowed to take place at 65°C for 20 min. Then, dDIP was performed as outlined above. Three volume of immunoprecipitated DNA were tested by multiplex PCR: 1 µl, 5 µl and 10 µl (out of the 30 µl). Best results were obtained with 5 µl.

Qualitative evaluation of immunoprecipitation efficiency by Multiplex PCR

The specific capture of TUNEL-labeled fragment (α and β) was assessed by multiplex PCR amplification (Qiagen Multiplex PCR Kit, Qiagen Inc.) using a Tgradient thermocycler (Biometra, Goettingen, Germany) and subsequent detection by agarose electrophoresis and ethidium bromide staining (see Figure 2b). As a negative control, i.e. a plasmid fragment that was not labeled by TUNEL, we used an internal fragment termed e (575 bp, position 1243 to 1817) to verify the specific capture of those labeled fragments. We followed the universal multiplex cycling protocol proposed by the manufacturer: initial activation at 95°C for 15 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min. Labeled DNA (before immunoprecipitation) was used as template for positive PCR control and negative PCR control was generated by the omission of DNA. For experiments using about 300 and 10 copies of the pcDNA3 plasmid as starting material, PCR products were concentrated five times (from 50 µl to 9–10 µl) with the MinElute PCR Purification Kit (Qiagen Inc.) before agarose

electrophoresis. The agarose gel was stained with SYBR Gold (Invitrogen) after electrophoresis for increased sensitivity.

Quantification of immunoprecipitated DNA by real-time PCR

Quantification of immunoprecipitated DNA was carried out by qPCR using either a Rotor-Gene RG-3000A or a Rotor-GeneRG-3000 (Corbett Research, Concorde, NSW, Australia) and the FastStart SYBR Green Master kit (Roche Applied Sciences). The sequence of the primers, expected amplicons size and annealing temperatures are summarized in Table A2-S1. For each amplicon, 2 µl of DNA sample was quantified in quadruplicate in a total of 10 µl of PCR mix containing 0.3 µM of primers, over 30 to 45 cycles of 15 s at 94°C, 30 s at 55°C to 60uC and 30 s at 72°C. Immunoprecipitation yield was expressed relative to input DNA according to $\%IP = (DNA_{IP}/DNA_{input}) \times 100$, where %IP is the percentage of immunoprecipitation, DNA_{IP} is the number of copies after immunoprecipitation measured by qPCR and DNA_{input} is the total number of copies before immunoprecipitation measured by qPCR.

Southern blots

Southern blots were performed as described previously [10]. Briefly, 5 µg of DNA were digested by BamHI and PvuII (MAT locus), PstI (PHO5 locus) or BglII (YcpHOCut4 plasmid), resolved on a 0.6% agarose gel and DNA was transferred onto a membrane under alkaline conditions. Nick-translated DNA probes were prepared by standard procedures using the following primers and incubated with the membranes: for the MAT locus, 5'-ATTCTTAGCATCATTCTTTGTTC-3' and 5'-TCCAATCTGTGCACAATGAAG-3', for the PHO5-HO locus, 5'-TCCGTGATGACGATGATTTG-3' and 5'-GTCAGTACCGGCTACTCTCT-3' and for YcpHOcut4 plasmid, 5'-ATGTTGAAGGAACAGCTGGG-3' and 59-GACCAATTAGACAATGGGAC-3'. The extent of DNA cleavage was determined by densitometry and expressed as the ratio of cut DNA to cut DNA + uncut DNA x 100.

For telomere length analysis, 5 µg of genomic DNA was digested either with the XhoI or PstI restriction enzymes, DNA was then analyzed by southern blotting, as described previously [8], using a probe covering part of the telomeric Y' fragment (5'-GGCCATTACTAGAAGAA-3' and 5'GGTACCCTCGTGTTATCTGCAGCG-3'). To evaluate the telomeres immunopre- cipitation efficiency by the dDIP technique, 1.5 µg, 2 µg and 2.5 µg of digested DNA before immunoprecipitation, representing 30%, 40% and 50% of the input DNA respectively, were loaded on gel.

Software

The following softwares were used: Enzyme X (version 3, Mek & Tosj), AmplifX (version 1.4.4, Nicolas Julien), Doc-ItLS Image Analysis Software (UVP, Upland, CA) and Roto-gene 6 (version 6.1, Corbett Research).

Results

Enrichment of DNA sequences in the vicinity of a unique PciI restriction site on the plasmid pcDNA3

To demonstrate the specificity and sensitivity of the DNA strand breaks capture, we first used a plasmid as a model representing a simple defined hotspot (Figure 2 and 3). To simulate a DSB, pcDNA3 was digested at a unique restriction site by the endonuclease PciI. The DNA ends were labeled by the incorporation of biotin-modified nucleotides (dUTP or dATP) at 3'OH termini by the TdT. The labeled DNA was further digested in fragments ranging from 4 to 720 bp by the endonuclease NlaIII which will generate probes of suitable size when applied to a genomic context. We first verified the presence of specific fragments α and β, flanking the PciI restriction site and also of the fragment ε, as an internal negative control, using multiplex PCR amplification as

illustrated in Figure A2-2. The results show specific capture of fragments α and β, whereas the fragment e is not present in the IP fraction and remains in the unbound fraction. Specific capture was successfully achieved with as low as 10 copies of pcDNA3 demonstrating the sensitivity of the capture method.

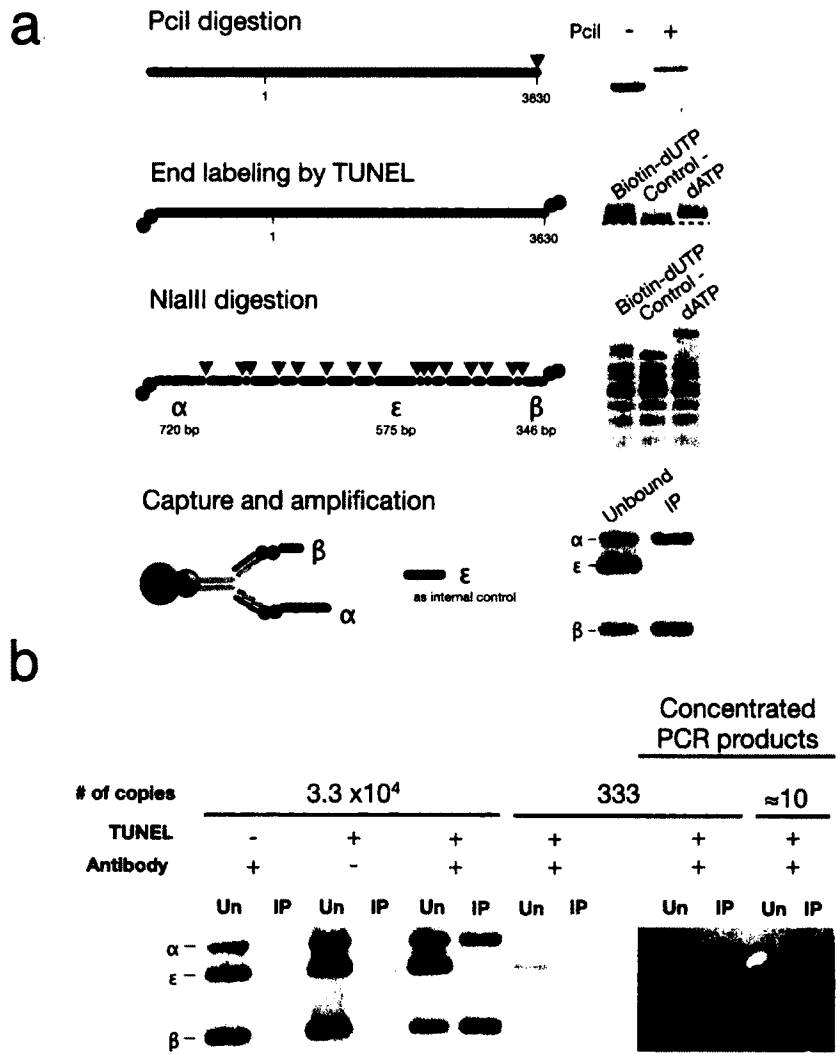

Figure A2-2. Enrichment of DNA sequences in the vicinity of a unique PciI restriction site present in the pcDNA3 plasmid.

(a) Step-by- step workflow of the dDIP for the in vitro plasmid model (b) Qualitative determination of enrichment by multiplex PCR using decreasing copy numbers of plasmid DNA as indicated. Un, unbound; IP, immunoprecipitation.

In order to establish the efficiency of immunoprecipitation, decreasing amounts of plasmids DNA (1 µg, 0.1 µg and 0.01 µg per IP) were immunoprecipitated, eluted and quantified by qPCR. Four amplicons where measured (Figure A2-3): two of these, amplicons B and C, are targeting the DNA sequences flanking the PciI restriction site, while two other amplicons, A and D, correspond to DNA sequences in the vicinity of the PciI restriction site but are separated by one or three NlaIII restriction sites and therefore serve as controls for specificity. Amplicons B and C were specifically enriched at each concentration of plasmid tested albeit with different yield ranging between 42% and 30% of total DNA for amplicon B and between 54.4% and 20.4% for amplicon C.

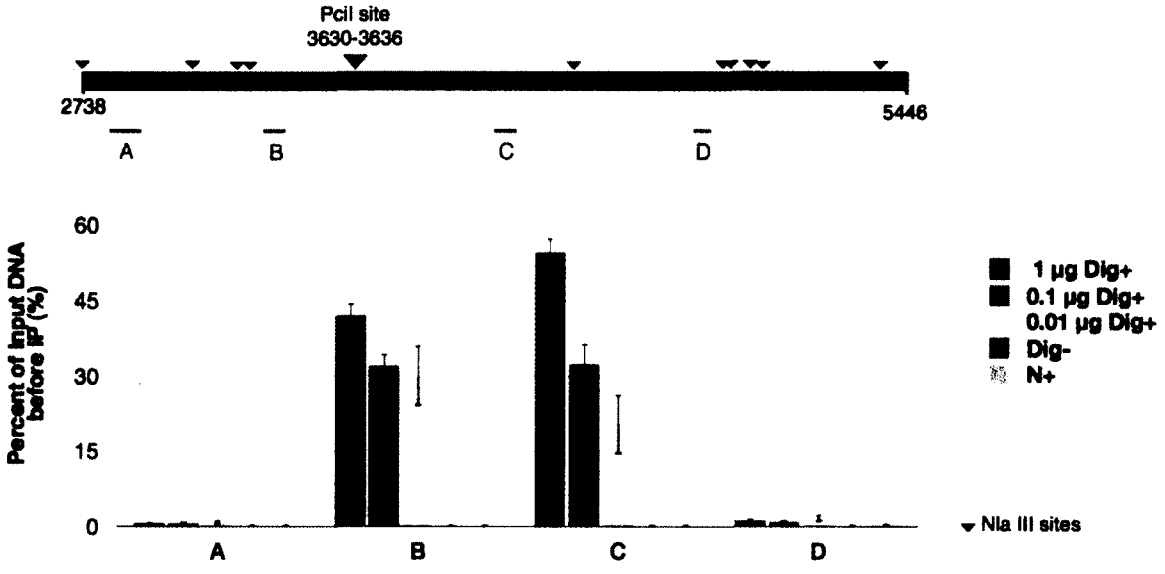

Figure A2-3. Quantification by qPCR of immunoprecipitated DNA sequences flanking the PciI restriction site of the plasmid pcDNA3.
Bars represent the average of two independent IP and error bars correspond to the standard deviation for qPCR measurements in triplicates. Dig+, PciI-digested pcDNA3 end-labeled with dATP, biotin-dATP and TdT; Dig-, PciI-digested pcDNA3 incubated with dATP and biotin-dATP without TdT; N+, intact pcDNA3 end-labeled with dATP, biotin-dATP and TdT.

Very few DNA sequences corresponding to amplicons A and D were immunoprecipitated in condition Dig+: between 0.6 and 0.8% for amplicon A and between 1% and 1.7% for amplicon D. This can be explained in part by incomplete digestion by NlaIII. Assuming that most of the pcDNA3 plasmids were digested by PciI, representing a double strand break in almost 100% of plasmids, we obtained an efficiency of capture between 20.4% and 54.4%, compared to 0 to 0.2% for the two negative controls representing the non-specific capture of DNA by the immunocomplex (Dig-) and the background DNA breaks from DNA purification and manipulations (N+).

As a first application of the dDIP technique in a genomic context, we applied the same strategy within a prokaryotic genome background. Overnight culture of *E. coli* DH5α transformed with pcDNA3 was embedded in 1% low melting agarose to minimize DNA fragmentation due to handling before labeling DNA ends. After lysis and protein digestion, DNA was digested in agarose plugs using PciI and labeled by TUNEL. After β-agarase digestion of the plugs, total DNA (genomic and plasmidic) was precipitated and further digested by NlaIII. Presence of the specific plasmid fragments was assessed by multiplex PCR amplification (see Figure A2-4). As for the experiment described above with the purified plasmid, specific capture of fragments α and β with limited background from fragment ε was obtained was again demonstrated.

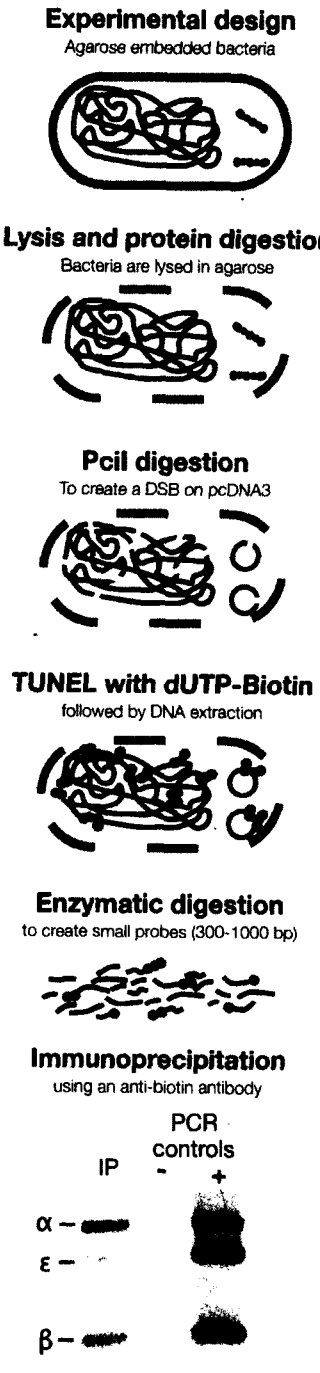

Figure A2-4. Multiplex PCR analysis of the captured DNA sequences in the vicinity of a unique PciI restriction site on the plasmid pcDNA3 transformed in DH5α *E. coli* and embedded in 1% agarose plugs.

Specific enrichment of three known HO sites within the genome of *S. cerevisiae*

We sought to determine whether specific capture of DNA strand breaks by dDIP could be achieved in the context of a whole eukaryotic genome. We therefore used the yeast *S. cerevisiae* as a model of in vivo genomic DNA breaks (reviewed in [11]). Specific capture of DNA sequences in the vicinity of HO-specific cleavage sites was demonstrated using the DFY046 strain in three different genomic context: first, using an inserted 60 bp HO site within the yeast *PHO5* gene; second, using the natural HO site within the mating-type locus (MAT) and finally using a 123 bp HO site within a transformed plasmid carrying the galactose-inducible HO endonuclease gene [7]. Specific enrichment of DNA sequences around the double-strand breaks was assessed by qPCR. Amplicons flanking the breaksites are identified by letters, A to P (see Table A2-S1 and Figure A2-5). Upon induction of the HO endonuclease expression by the addition of galactose in the media, a DSB is created providing an ideal experimental model from which the capacity of the dDIP technique to specifically capture genomic hotspots can be established. Capture of the labeled restriction fragment harboring the HO site but not adjacent fragments harboring no such site is therefore expected. Enrichment of fragments harboring an HO-induced DSB can then be compared to these adjacent HO-free sites. Negative controls include non-specific binding to the immunocomplex in the induced but not end-labeled state (I-) as well as background DNA damage due to extraction and DNA breaks not related to the HO endonuclease (N+). Finally, enrichment of immunoprecipitated DNA sequences harboring the three HO sites can be expressed relative to the cutting efficiency of the total HO activity as determined by southern blot analyses.

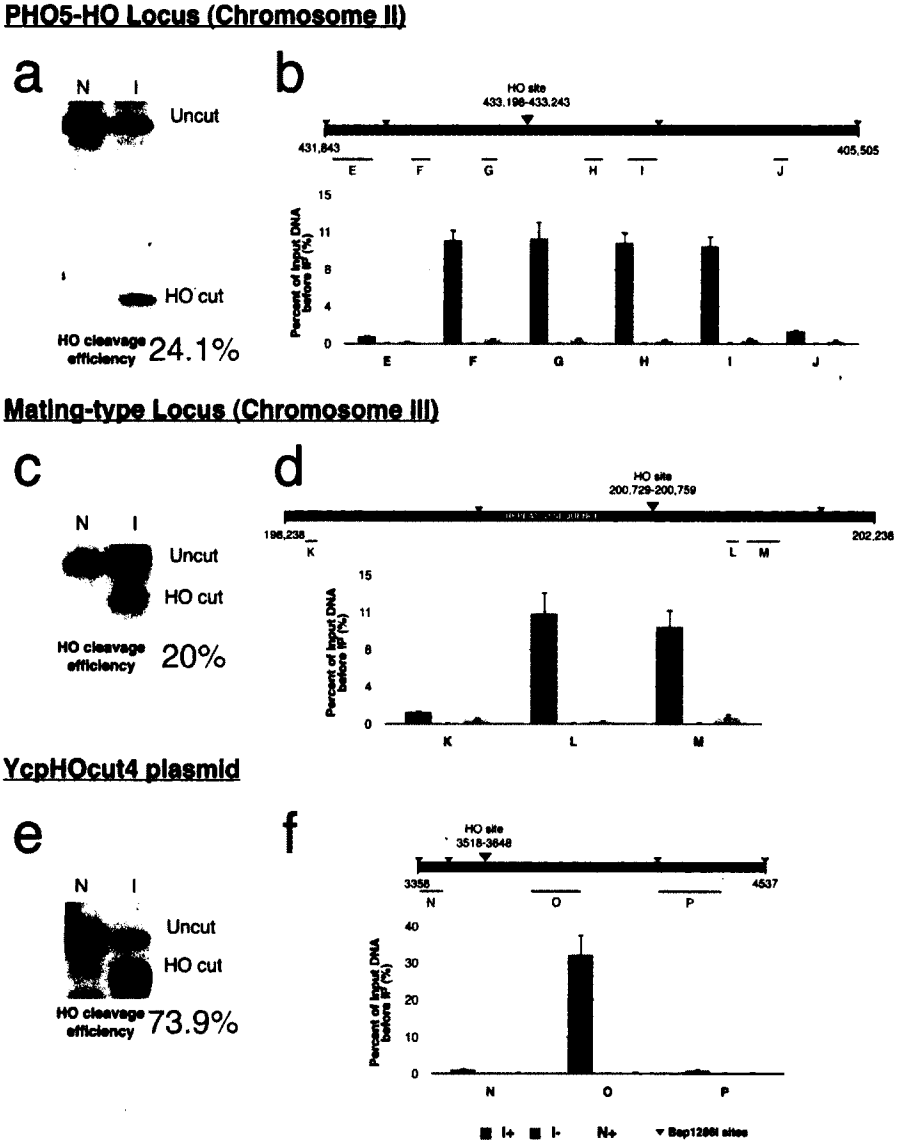

Figure A2-5. Enrichment of DNA sequences in the vicinity of three induced DSBs cleaved by the induced HO endonuclease in the yeast S. cerevisiae.
(a) Southern blot analysis of the HO site cleavage efficiency at the recombinant PHO5-HO locus (b) Immunoprecipitation of DNA sequences flanking the HO cut site within PHO5-HO, digested by Bsp1286I and quantified by qPCR. (c) Southern blot analysis of the HO site cleavage efficiency at the mating-type locus. (d) Immunoprecipitation of DNA sequences flanking the HO cut site of the Mating-type locus, digested by Bsp1286I and quantified by qPCR. (e) Southern blot analysis of the HO site cleavage efficiency within the YcpHOcut4 locus (f) Immunoprecipitation of DNA sequences flanking the HO cut site of the YcpHOcut4 plasmid, digested by Bsp1286I and quantified by qPCR. Error bars represent standard deviation for 3 independent IP. I+, DNA from HO-induced cells end-labeled with dATP, biotin-dATP and TdT; I-, DNA from HO-induced cells end- labeled with dATP, biotin-dATP without TdT; N+, DNA from uninduced cells end-labeled with dATP, biotin-dATP and TdT.

Upon galactose induction and HO endonuclease synthesis, all three HO cleavage sites were efficiently captured by the dDIP technique in sharp contrast to the virtual absence of immunopre- cipitated DNA sequences represented by amplicons laying outside the HO cleavage sites (Figure A2-5b, d, f). The later are sequences that were separated from the initial TdT-labeled break site by subsequent restriction digestion using a frequent cutting enzyme Bsp1286I. Similarly, background DNA damage, revealed by the N+ immunoprecipitates remains virtually undetected for the three tested loci.

At the PHO5-HO locus, the two DNA sequences immediately flanking the induced HO cleavage site and represented by amplicons F to I, were captured with an average efficacy of 10.3% over total DNA (before immunoprecipitation) representing a 10-fold enrichment over the amplicons E and J not harboring the HO site (Figure A2-5b). Similar observations were made for the MAT locus where amplicons harboring the HO sites (L, M) were enriched to the same extent compared to the unlabeled fragment represented by amplicon K (Figure A2-5d). Finally, we observed a corresponding enrichment of the DNA sequences flanking the extrachromosomal HO cleavage site of the plasmid YcpHOcut4 (Figure A2-5f). In this case, the amplicon O, corresponding to the fragment adjacent to the break, was enriched about 32-fold upon induction compared to fragment N and P. The absence of non- specific DNA binding to the immunocomplex is indicated by the near absence of detected amplicons in all induced but not end- labeled immunoprecipitates throughout the experiments. Considering the limited extent of HO cleavage demonstrated by the southern blots as seen in Figure A2-5, 42% of available DNA termini at break sites were immunoprecipitated at the PHO5-HO locus, whereas 52% and 43% were immunoprecipitated at the MAT locus and the YcHOcut4 locus respectively.

Enrichment of yeast telomeres

Telomeres constitute natural DNA double-strand breaks and should therefore be captured by the dDIP technique. The presence of telomeres in immunoprecipitates of labeled non- induced DFY046 DNA was determined using two endonucleases known to cut once in the conserved telomere proximal Y' repeat elements. As shown in Figure A2-6 and Figure A2-S1, we obtained similar immunoprecipitation efficiency as shown for HO sites, representing 40 to 50% of the telomere termini available. The immunoprecipitated telomeric sequences appear as a smear due to the end-labeling of the TdT and the gel-shift caused by the added biotin hapten. No telomere DNA was detected in the N- lane measuring the non-specific binding to the immunocomplex.

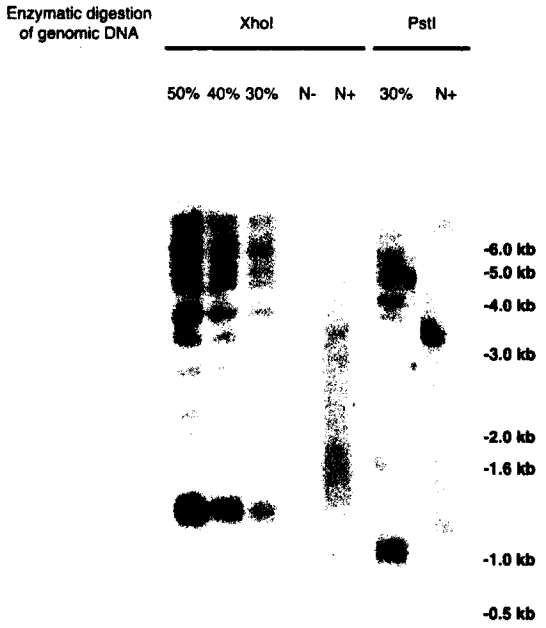

Figure A2-6. dDIP enrichment of yeast telomeric DNA evaluated by Southern blot. Extracted yeast DNA was first digested by XhoI or PstI, two enzymes cutting once in the conserved telomere proximal Y' repeat element giving a ≈1.2 kb and ≈1.0 kb terminal restriction fragment respectively.
A probe covering part of the telomeric Y' fragment including the terminal 0.35 kb TG1-3 repeats was used to reveal the capture of telomeric DNA. To evaluate the telomeres immunoprecipitation efficiency by the dDIP technique, 30%, 40% and 50% of the input DNA before immunoprecipitation was applied to the gel. N+, DNA from uninduced cells end-labeled with dATP, biotin-dATP and TdT. N-, DNA from uninduced cells end-labeled with dATP, biotin- dATP without TdT.

Discussion

We have provided the technical details for specific and sensitive capture of DNA sequences at strand breaks and shown its application to a genomic context in vivo. We simultaneously enriched DNA sequences flanking three different induced DSBs in the yeast S. cerevisiae as a model of multiple genomic hotspots. Furthermore, we have demonstrated that dDIP can be used to enrich telomeres with high efficiency.

In association with microarray technologies or high throughput next-generation sequencing approaches, the dDIP could be used to map the distribution of DNA strand breaks on a genome-wide scale without prior knowledge of sensitive loci. In a single experiment, genome-wide mapping of DNA breaks can be achieved with nucleotide level resolution when combined with next-generation sequencing methods. Indeed, owing to the tailing activity of the TdT, one can reach the nucleotide resolution of primer-specific methods such as LM-PCR and thus establish the exact position of break sites for all labeled sequences at once. When combined to real-time PCR, the enrichment of known specific loci can be precisely determined, as demonstrated in this work.

Other related DNA immunoprecipitation techniques, such as chromatin immunoprecipitation (ChIP), usually display low capture efficiencies often down to 1% of the input of protein/ DNA complexes and rarely higher than 20%. As shown in the present paper, immunoprecipitation efficiency of about 40–50% of available DNA termini was achieved with the dDIP technique, which suggests a better efficiency most suitable for the capture of rare damage events. dDIP is expected to be far more precise than ChIP using antibodies against DNA repair proteins or histone post-translational modifications, as they are often masked at the site of DNA damage (due to complexing) or found associated with break sites for short periods of time. In addition, these factors may be spanning long distances over break sites. One such example is the DSB

biomarker γH2AFX, the active form of the histone variant H2AFX (H2A histone family, member X), that is known to spread up to one megabase from the break site in mammalian cells [12]. Thus, dDIP allows for the precise mapping of the DSB with much greater resolution.

We have also shown that DNA extracted with commercially available kits, although not optimal because of non-specific DNA damage due to the extraction process, is suitable for a sensitive enrichment of DNA damage since 5 x 10^7 haploid copies of the yeast genome is sufficient. DNA breaks must ideally be labeled before the genomic DNA extraction to decrease the background levels of DNA damage induced from handling. When this cannot be achieved, as in the case of the yeast S. cerevisiae genomic DNA reported in this work, kits that purify large DNA fragments (50–100 kb) should be used. Therefore, DNA damage from any source can be mapped provided that DNA integrity is maintained during extraction.

The length of DNA sequences may be adapted to fit the experimental goals as larger DNA fragments (up to 9,400 bp) were successfully tested in this work (data not shown). As demonstrated in this paper, reduction in fragment size can be achieved enzymatically, by a frequent cutting restriction enzyme, but also physically, by sonication or nebulization (see supplementary methods and Figure A2-S2 for an example of sonication of the PHO5-HO locus). In this work, enzymatic fragmentation of genomic DNA was preferred as it provided defined boundaries outside of which specificity could be established from the virtual absence of capture.

Recently, concomitant with our efforts to further refine this method before publication, new approaches were proposed to map genome-wide DNAseI-hypersensitive regions and DNA breaks. One such method, known as DNAse-ChIP, was used to map open chromatin regions using an indirect approach where unbound DNA was cleaved by DNaseI and ligated to a biotinylated linker [13,14,15]. An another approach very similar to that described in the present report was used to map nuclear receptor-dependant tumor translocations by the androgen receptor, although impor-

tant technical considerations reported in the present paper to improve efficiency were not provided [4]. For instance, we demonstrated the high efficiency of the dDIP technique mainly provided by the powerful yet versatile TdT end-labeling of DNA termini and the improved tailing of these breaks by the addition of unmodified dATP in the labeling mix to increase the number of biotin-modified nucleotides and to ensure an optimal spacing of the haptens [16], resulting in enhanced efficiency of capture by the anti-biotin antibody. We trust that these improvements over previously reported approaches may increase chances of finding rare events and more robust identification of DNA break sites.

The terminal transferase can efficiently add modified nucleotides to any free 3'OH DNA terminus such as a 3' overhang, blunt or 5' overhang extremities of a double-strand break, with a preference for 3' overhang termini, but also single-strand breaks and single-strand DNA (ssDNA). In this paper, we deliberately chose a suboptimal substrate for the in vitro plasmid model as the endonuclease PciI generates a four-nucleotide 5' overhang without altering end-labeling or immunoprecipitation efficiencies. TdT may have more difficulty adding the first nucleotides to a single-strand break, a 5' overhang or blunt terminus than a 3' overhang terminus, but this may not constitute a strong bias considering the long tails that we generate using a mix of dATP and biotin- modified dATP. We have also achieved similar capture yield in the vicinity of a single-strand break compared to a double-strand break as demonstrated with the plasmid model (see supplementary methods, Figure A2-S3 and Table A2-S2). In addition, we have obtained similar results with the homing endonuclease HO that creates two-nucleotide 3' overhang termini, the substrate of choice for the TdT. We have therefore experimentally demonstrated that DNA sequences with single-strand breaks or any type of double-strand breaks can be enriched by dDIP.

The dDIP technique can be applied to a variety of studies aimed to assess genomic integrity in both normal and pathological processes. DNA strand breaks are required for normal processes such as meiotic recombination [17], lymphocyte development [18], cell differentiation [19], transcription [20] and were associated

with chromatin remodeling of spermatids as demon- strated by our group [21]. They also occur in response to exogenous stress such as oxidation, radiation, drugs and environmental pollutants or in degenerative processes. Damaged DNA immunoprecipitation can also be a very versatile method as it has the potential to map several types of DNA damage from small amount of DNA (less than 1 μg) in various genomic and experimental contexts, provided that these lesions can be converted into DNA strand breaks. Our cells possess several enzymes that can convert or remove all kinds of DNA damage to ensure the genome's integrity. Inevitably, these mechanisms must go through steps that create a 3'OH terminus as it is essential for the activity of polymerases and ligases involved in DNA repair. Therefore, in addition of single- and double-strand breaks, dDIP can enriched other types of DNA damages that triggers in vivo repair. Furthermore, these DNA damage can be converted in vitro to a 3'OH termini by commercially available enzymes. For instance, cyclobutane pyrimidine dimers caused by UV irradiation can be converted by the T4 endonuclease V to a strand break with an atypic nucleotide (3'phospho terminus) [22] that can be removed by the exonuclease activity of a DNA polymerase allowing its capture by dDIP.

In summary, the important experimental details provided in this paper aim to provide a reproducible method for the capture of DNA strand breaks. It is a simple, fast and economic approach applicable to a genome-wide scale. This new technique should prove highly useful to the study of several types of cellular processes where profiling of damage will be most needed.

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Supplementary Methods and Results

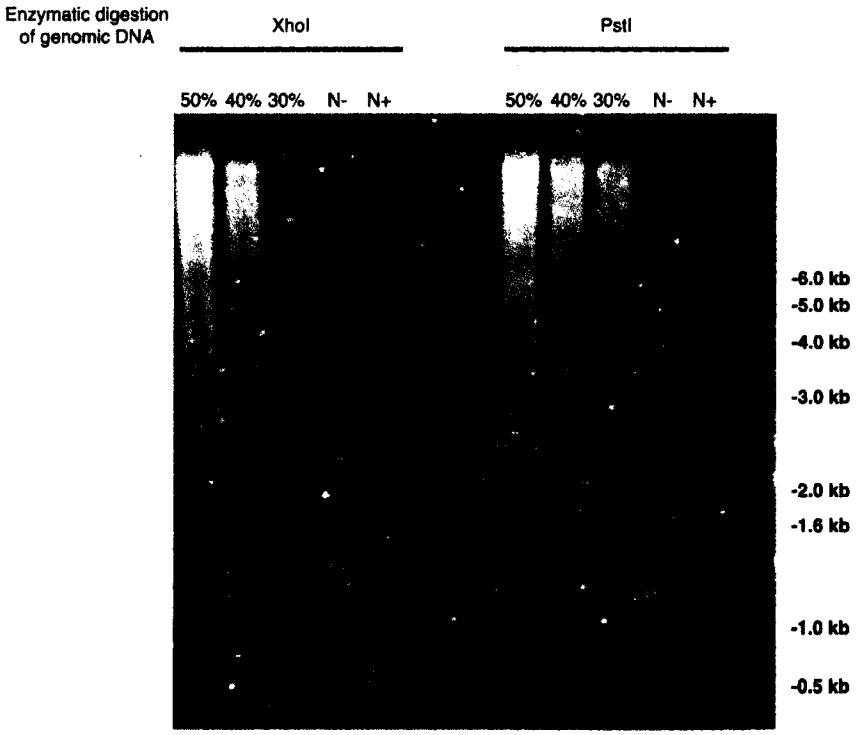

Figure A2-S1. Ethidium bromide stained agarose gel of enriched telomeric DNA before southern blot analysis.

Extracted yeast DNA was first digested by XhoI or PstI, two enzymes cutting once in the conserved telomere proximal Y' repeat element giving respectively a ≈1.2 kb and ≈1.0 kb terminal restriction fragment. To give an indication of the telomeric immunoprecipitation efficiency by the dDIP technique, 1.5 µg, 2 µg and 2.5 µg of digested DNA before immunoprecipitation, representing 30%, 40% and 50% respectively of the input DNA, were loaded on gel.

Table A2-S1 Primers used for PCR applications.

Name	Test	Location	Temperature of annealing (°C)	Product size (bp)	Forward primer sequences	Reverse primer sequences
α	Multiplex PCR	pcDNA3 plasmid	60	720	5'-TCCGCTCACAATTCCACACAAC-3'	5'-TACCGCCTTTGAGTGAGCTGAT-3'
β	Multiplex PCR	pcDNA3 plasmid	60	346	5'-CAGCAAAAGGCCAGGAACCGTAA-3'	5'-TTTCGTTCCACTGAGCGTCAGA-3'
ε	Multiplex PCR	pcDNA3 plasmid	60	575	5'-TATGGCTTCTGAGGCGGAAAGAAC-3'	5'-TGGGGACTTTCCACACCCTAACT-3'
A	qPCR	pcDNA3 plasmid	59	182	5'-TGATATTGCTGAAGAGCTTGGCGG-3'	5'-TCGAAATCTCGTGATGGCAGGTTG-3'
B	qPCR	pcDNA3 plasmid	59	134	5'-ATTCCACACAACATACGAGCCGGA-3'	5'-TAATGCAGCTGGCACGACAGGTTT-3'
C	qPCR	pcDNA3 plasmid	59	127	5'-ACAGAGTTCTTGAAGTGGTGGCCT-3'	5'-TGGTTTGTTTGCCGGATCAAGAGC-3'
D	qPCR	pcDNA3 plasmid	59	92	5'-TTGTTGCCATTGCTACAGGCATCG-3'	5'-TGTAACTCGCCTTGATCGTTGGGA-3'
E	qPCR	PHO5	58	275	5'-GATGAGCCTTACCCACCCATGATTTC -3'	5'- TTGGCGATGGGATAACCAAGGAAC-3'
F	qPCR	PHO5	58	131	5'-TGCGAGAAACGTGACCCAACTT -3'	5'- TGGGGACTGACATCAGGTCAACAT-3'
G	qPCR	PHO5	58	109	5'- ACACGTGGGACTAGCACAGACTAA-3'	5'- GGGTATATGCCTTGCCAAGTAAGGTG-3'
H	qPCR	PHO5	58	127	5'-TCATGTCCTGCTTGGGACTACGAT -3'	5'-CGTCAGTTGAGGTCAAGTTCAAACCC-3'
I	qPCR	PHO5	57	204	5'-GTCGGTTCCAACTTGTTCAATGCC -3'	5'- AGGAACGTACCAGGATCTGTGGAA-3'
J	qPCR	PHO5	58	99	5'- GGAGAGTTAGCCGATGTTGCCAAA-3'	5'- ACCATAGTCGCCAGGGAAAGAGAA-3'
K	qPCR	MAT	58	92	5'- GTCAGTTGCACCGCACAATTCATC-3'	5'- AAAATAAACCGCCCCTGGACTACG-3'
L	qPCR	MAT	56	87	5'-AACGAATTGGCTATACGGGACGGA-3'	5'-GGGCAGTTTACCTTTACGGTTTGT-3'
M	qPCR	MAT	55	227	5'- GCTAGTTACCTTCGGCTTCACA-3'	5'-TGGAAACACCAAGGGAGAGAAG-3'
N	qPCR	YcpHOcut4 plasmid	56	80	5'-TTGTTTCGGCGTGGGTATGGT-3'	5'- CAAGGAATGGTGCATGCAAGGAGA-3'
O	qPCR	YcpHOcut4 plasmid	58	170	5'-TCATGCAACTCGTAGGACAGGT-3'	5'-TTCTCGCCGAAACGTTTGGT-3'
P	qPCR	YcpHOcut4 plasmid	58	215	5'-ATGGAACGGGTTGGCATGGATT-3'	5'-GACGCGATGGATATGTTCTGCCAA -3'

Enzymatic digestion vs. sonication

To compare enzymatic digestion and sonication, we performed dDIP as specified previously with the in vivo yeast model (*S. cerevisiae*). Sonicated samples were prepared as follows: after end-labeling and subsequent reaction arrest with EDTA, the samples were directly sonicated in 1.5 ml eppendorf in cool water using a Sonicator 4000 coupled sonication cup horn (Misonix Sonicators, Newtown, CT) set at an amplitude of 20 for 6 cycles of 30 seconds with 30 seconds pauses between cycles. DNA was then precipitated to remove excess nucleotides and TdT and resuspended in citrate buffer. The efficiency of sonication was verified by agarose gel electrophoresis; we observed a smear of DNA fragments between 300 and 1,500 bases (data not shown). Two independent immunoprecipitations were done and enrichments are presented in Figure A2-S2.

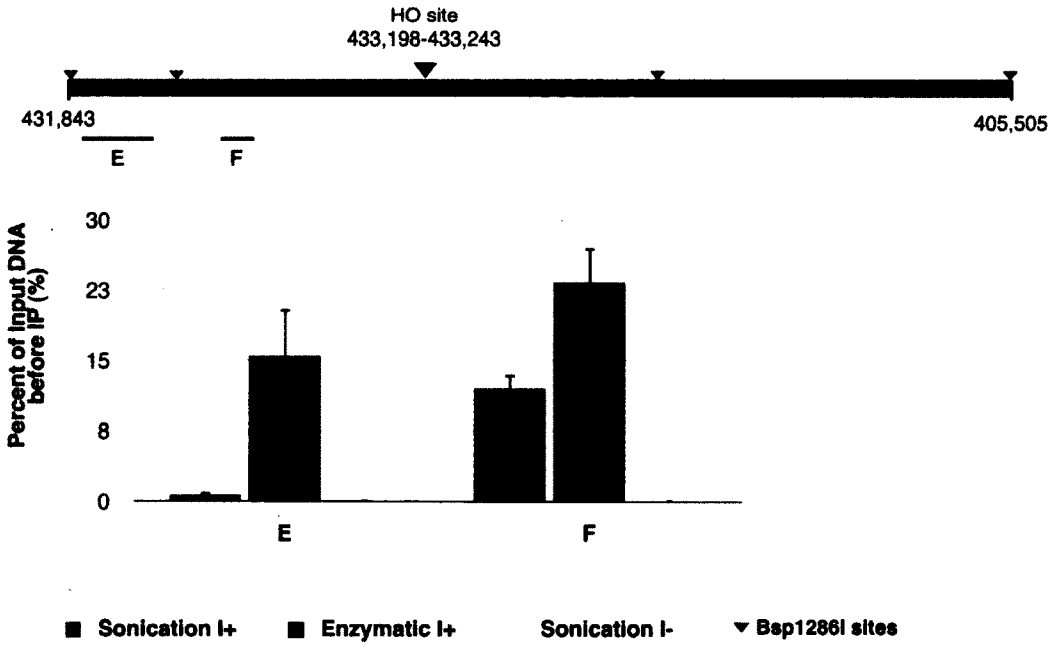

Figure A2-S2. Comparison between enzymatic fragmentation of yeast genomic DNA and sonication measured by the enrichment of DNA sequences around the PHO5-HO site.
I+, DNA from HO-induced cells end-labeled with dATP, biotin-dATP and TdT; I-, DNA from HO-induced cells and unlabeled by the omission of TdT.

As expected, we observed a decline in enrichment of sonicated samples between amplicons E and F due to distance from the break and the generated fragment size (300 to 1,500 bases), whereas enzymatic digestion resulted in similar enrichments and similar pattern as presented in Figure A2-5. However, sonication samples presented larger standard deviations that may be due to uneven sonication within the samples. Overall, sonication and enzymatic digestion are suitable options to fragment genomic DNA for dDIP.

Enrichment of DNA sequences in the vicinity of single-strand breaks on the plasmid pcDNA3

To demonstrate the capacity of dDIP to enrich sequences carrying single-strand breaks or nicks, we used the *in vitro* pcDNA3 plasmid model. First, the plasmid pcDNA3 was nicked at three sites by the nicking endonuclease Nt.BspQI (New England Biolabs) according to the manufacturer's recommendations and referred to as Nt+; as a negative control, the enzyme was omitted for the DNA sample (Nt-). As a second control, referred to as Nt+lig, we sealed the nicks using the T4 DNA ligase (New England Biolabs) at room temperature for 30 minutes. DNA samples were then purified using a Qiagen PCR purification kit (see Figure A2-S3a, lanes 1-3). As previously described in section "Plasmid preparation and end-labeling" of the manuscript, DNA samples were end-labeled, precipitated and digested by NciI (New England Biolabs). Before immunoprecipitation, DNA samples were resolved on a 0.8% agarose gel to verify the addition of nucleotides by the TdT to the specific nicked fragments (see Figure A2-S3a, lanes 4-6, the red arrows indicate the two nicked fragments). The enrichment of specific DNA fragments was measured by qPCR.

Demonstrated in Figure A2-S3a, the great majority of Nt.BspQI digested plasmids (close to 100%) were circularized (see lanes 2-3) and after end-labeling and NciI digestion, almost all of the two nicked fragments, one of 954 bp with one single-strand break and the other of 613 bp nicked twice, shifted in Nt+ sample because of

end-labeling (see lane 5). The T4 DNA ligase efficiently sealed the single-strand breaks of the N+lig sample as shown in lane 6, where no shift of these two DNA fragments was observed. The nicked plasmid fragments, represented by amplicons A and B, were successfully enriched in Nt+ sample compared to other plasmid sequences (amplicons C and D) separated by one or two NciI restriction sites, but also compared to the two controls, Nt- and Nt+lig for these specific fragments (see Table A2-S2 and Figure A2-S3b). A minuscule carryover enrichment (60 to 86 fold) can be observed in amplicons C and D in sample Nt+ compared to Nt- (0.06222% and 0.1004% for Nt+ compared to 0.001014% and 0.001168% for Nt-) due to incomplete NciI digestion; less 0.002% of plasmids were not completely digested by NciI according to these results demonstrating again the sensitivity of dDIP (see Table A2-S2). We obtained similar immunoprecipitation efficiency (42.6% for amplicon A and 77.4% for amplicon B) as for double-strand breaks enrichment with the same *in vitro* model (42% for amplicon B and 54.4% for amplicon C, see Figure A2-3). We therefore conclude that sequences carrying single-strand breaks can also be enriched by dDIP with similar effiencies as for sequences flanking double-strand breaks.

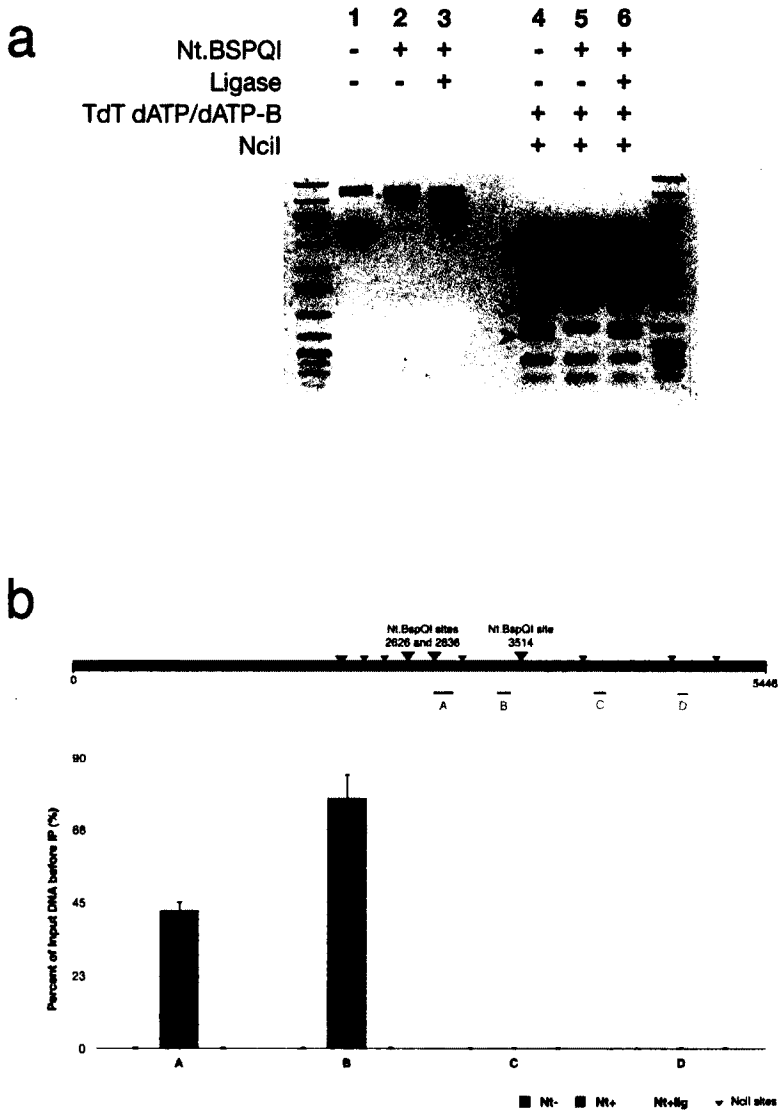

Figure A2-S3. Enrichment of DNA sequences in the vicinity of single-strand breaks on the plasmid pcDNA3.

(a) Ethidium bromide stained agarose gel of different steps of the workflow for the enrichment of nicked DNA sequences using the in vitro pcDNA3 plasmid model. Lanes 1–3; pcDNA3 plasmid DNA after ligase reaction, prior to end-labeling. Lanes 4–6; NciI digested and end-labeled pcDNA3 plasmid DNA, prior to immunoprecipitation. The red arrows indicate the two frements carrying one or two single-strand breaks. DNA ladder; GeneRuler^{tm} 1 kb Plus DNA ladder (Fermentas). (b) Quantification by qPCR of immunoprecipitated DNA sequences flanking the Nt.BspQI restriction sites within pcDNA3. Bars represent the average of two independent IP and error bars correspond to the standard deviation for qPCR measurements in quadruplicates. Nt-, pcDNA3 plasmid not digested by Nt.BspQI; Nt+, pcDNA3 plasmid digested by Nt.BspQI; Nt+lig, pcDNA3 plasmid digested by Nt.BspQI and ligated.

Table A2-S2 Enrichment of DNA sequences in the vicinity of single-strand breaks on the plasmid pcDNA3.

	Amplicon A	Amplicon B	Amplicon C	Amplicon D
Nt-	0.001965 ± 0.000158 %	0.002600 ± 0.000443 %	0.001014 ± 0.000132 %	0.001168 ± 0.000147 %
Nt+	42.59 ± 2.84 %	77.39 ± 7.01 %	0.06222 ± 0.0130 %	0.1004 ± 0.0514 %
Nt+lig	0.004412 ± 0.000300 %	0.005330 ± 0.000592 %	0.002676 ± 0.000290 %	0.002130 ± 0.000244 %

Percentage of enrichment based on two independent immunoprecipitation (average 6 standard deviation %). Nt-, pcDNA3 plasmid not digested by Nt.BspQI; Nt+, pcDNA3 plasmid digested by Nt.BspQI; Nt+lig, pcDNA3 plasmid digested by Nt.BspQI but followed by a T4 DNA ligase reaction.

Chapitre 3 : applications aux cellules eucaryotes supérieures et adaptations à d'autres dommages

Avant-propos

Le troisième objectif de mon doctorat avait pour but d'appliquer la nouvelle méthode de cartographie des cassures, mise au point grâce au modèle de la levure *S. cerevisiae*, à l'étude des cassures bicaténaires transitoires des spermatides allongeantes. La levure *S. cerevisiae* offrait plusieurs avantages notamment un petit génome, une séquence précise où l'on pouvait induire une cassure bicaténaire, mais possédait aussi quelques défauts majeurs dont le principal était la nécessité d'extraire l'ADN avant de pouvoir marquer les cassures étant donné que la levure possède une paroi cellulaire beaucoup résistante que celles des cellules de mammifères.

Cette difficulté amenait de nombreuses cassures non-spécifiques créant un bruit de fond et qui variait d'une expérience à l'autre en fonction de la « douceur » de l'extraction d'ADN. Nous savions par cette observation que l'utilisation de kits d'extraction d'ADN génomique commerciaux amèneraient un bruit de fond encore plus important avec des cellules possédant un génome plus volumineux, tel que celui de la souris et de l'humain. De plus, pour avoir passé plusieurs mois, voire plusieurs années, à tenter un marquage TUNEL sur des cellules germinales en suspension, il était difficile d'utiliser ce type de protocole avec les spermatides car elles finissent inévitablement par s'agréger pendant les centrifugations nécessaires aux lavages.

Il fallait donc mettre au point une approche qui n'engendrerait pas ou le moins possible de cassures avant le marquage de la TdT et qui ne se ferait idéalement pas en suspension, du moins avec le moins possible de centrifugation. Après une tentative infructueuse avec une approche utilisant la technique « squash »(Kotaja et al., 2004),

nous avons opté pour une méthodologie en « plug » d'agarose. De plus, nous avons utilisé un modèle de cassure bicaténaire inductible avec la méganucléase I-SceI (ou *homing endonuclease* en anglais), provenant des mitochondries de la levure *S. cerevisiae*, dont le site de reconnaissance n'est pas présent ni chez la souris ni chez l'humain. En introduisant la séquence de reconnaissance de I-SceI par génie génétique dans des cellules HeLa, il était ainsi possible d'induire une coupure bicaténaire unique dans le génome humain par transfection et optimiser l'enrichissement de cette séquence par dDIP.

Ce chapitre contient le résumé d'une première tentative de cartographie des cassures chez les spermatides, la mise au point d'un protocole fonctionnel de dDIP sur des cellules d'eucaryotes supérieurs, l'élaboration d'une méthode unique de purification par FACS de cellules germinales mâles, et d'une deuxième tentative de cartographie des cassures avec des cellules triées au FACS. Avec cette nouvelle méthode de purification (voir le manuscrit en Annexe 4), il sera possible d'obtenir suffisamment de spermatides allongeantes pour établir une cartographie des cassures bicaténaires. De plus, j'aborderai l'adaptation de la technique dDIP pour établir la distribution à l'échelle d'un génome de dommages autres que des cassures, soit les dommages oxydatifs et ceux causés par la radiation UV. Finalement, j'élaborerai sur les technologies de séquençage de troisième génération qui permettront d'atteindre le plein potentiel de l'approche dDIP pour une cartographie à l'échelle génomique des dommages à l'ADN et ce, à la résolution nucléotidique.

Frédéric Leduc est l'auteur des concepts décrits dans ce chapitre. La majorité de expériences ont été exécutées par Frédéric Leduc, avec le support de la professionnelle de recherche du laboratoire Boissonneault, Mélina Arguin. Tout autre apport par d'autres membres du laboratoire ou de d'autres laboratoires sera spécifié au cours du chapitre.

dDIP sur squash : première tentative à l'échelle d'un génome

Malgré de nombreuses tentatives de purification des spermatides par cytométrie en flux (FACS) au cours des deux premières années de doctorat, nous n'avions pas de technique appropriée pour séparer des spermatides allongeantes subissant le remodelage chromatinien. J'ai aussi tenté de purifier avec un certain succès les spermatides sur un gradient de BSA (gradient 2%-4% de BSA), une méthode appelée STAPUT ou CELSEP (Janulis, Bahr, Hess, & Bunick, 1996; Wykes & Krawetz, 2003a). Bien que cette approche procurait de nombreux avantages, tels que des populations purifiées de plusieurs millions de cellules vivantes et une séparation relativement efficaces des populations, il était impossible d'obtenir une population de spermatides allongeantes (étapes 9-13) à plus de 50% de pureté, étant trop intermédiaire entre les spermatides rondes (étapes 1-8) et les spermatides condensées (étapes 14-16). Nous avons alors considéré de sélectionner toutes les fractions contenant des cellules haploïdes ne présentant aucune contamination de d'autres types cellulaires, mais nous n'avions aucune approche qui permettrait de marquer les cassures sans perdre la grande majorité des cellules, ou douter d'un marquage égal de toutes les cellules. De plus, nous avons mis au point et évalué la possibilité d'utiliser la capture par microdissection laser sur des sections de testicules, que nous pouvions facilement marquer au TUNEL. Bien que cette approche était très précise et offrait une pureté inégalée, le nombre de cellules récoltées par journée de microdissection laser était bien insuffisant pour des applications de cartographie des cassures par dDIP.

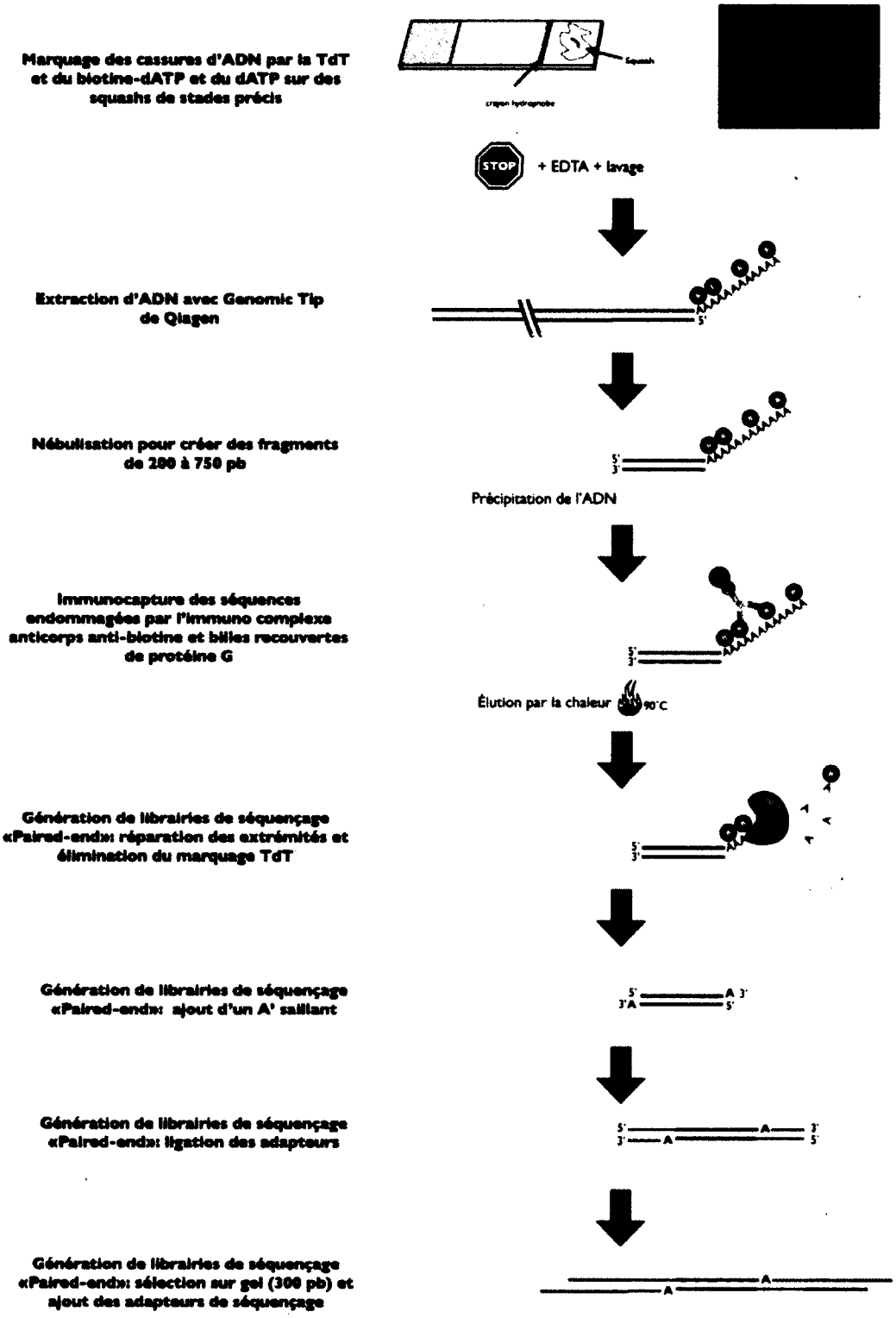

Figure A3-1. Schéma expérimental pour le dDIP utilisant des préparations « squash » de tubules de stades choisis.

Nous avons donc opté pour un compromis en utilisant une approche soustractive (voir Figures A3-1 et A3-2) utilisant des préparations « squash » de tubules de stades précis marquées au TUNEL tel que décrit dans le premier chapitre de cette thèse et dans l'article suivant (Kotaja et al., 2004). Dans un premier temps, nous avons effectué un enrichissement de régions endommagées provenant de préparations squash de testicules de souris immatures de 23 jours dont le développement germinal ne dépassait pas le stade des spermatocytes pachytènes(Godmann et al., 2007) (en bleu à la Figure A3-2), représentant les cassures dues à la mitose des spermatogonies et l'enjambement de la méiose. Puis, nous avons sélectionné les tubules « pâles » correspondant aux stades IX à XII de souris matures (plus de 42 jours) et effectué le dDIP pour générer une librairie de séquençage représentant les régions endommagées de la méiose et du remodelage de la chromatine des spermatides allongeantes (en jaune à la Figure A3-2). Ainsi, les régions endommagées retrouvées dans les deux librairies de séquençage correspondraient à des cassures liées à la méiose (en vert à la Figure A3-2); les autres régions non-communes représenteraient les cassures liées à la mitose pour la première librairie ou au remodelage de la chromatine des spermatides dans la deuxième.

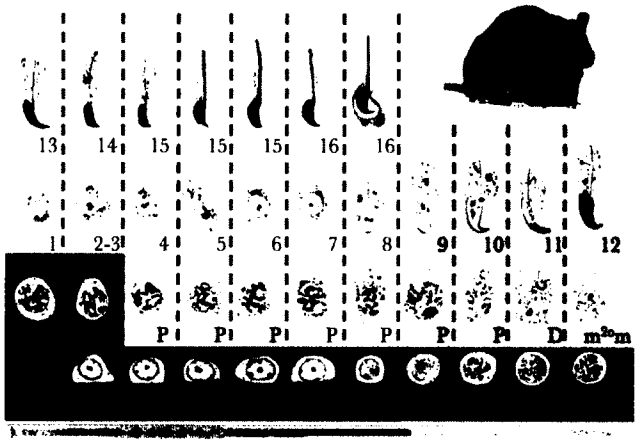

Figure A3-2. Stratégie soustractive des librairies de séquençage pour déterminer les cassures appartenant à la mitose des spermatogonies, l'enjambement de la méiose et les cassures associées au remodelage de la chromatine, utilisant des préparations « squash » de tubules de stades choisis.

Pour mettre au point un protocole de génération de librairies de séquençage de type Solexa (Illumina inc.) et approfondir mes connaissances en séquençage de deuxième génération et en bio-informatique, j'ai effectué un stage de 5 mois dans le laboratoire de Professeur Stephen A. Krawetz (Wayne State University, Détroit, Michigan, USA). À mon retour, nous avons effectué l'expérience à partir des squashs et généré les librairies indexées de séquençage. Le séquençage a été effectué sur un séquenceur Illumina GAIIx au centre de génomique de Wayne State University. Ce premier séquençage a donné des lectures de très basse qualité, la plupart étant mises de côté par le premier filtre de qualité du séquenceur. Ce problème est possiblement expliqué par une quantité inappropriée d'ADN déposée sur la puce de séquençage par le technicien ou tout autre problème lors de la formation des amas (*clusters*). Ayant tenté une analyse des résultats de séquençage qui comportait une qualité appropriée (une faible proportion) et la soustraction des librairies, nous avons vite réalisé que cette stratégie grandement utilisée avec la technologie des biopuces s'appliquait difficilement au séquençage de deuxième génération étant donné la grande diversité des lectures (vs. les sondes établies d'une biopuce).

Nous nous sommes donc tournés vers une stratégie que nous avions déjà utilisée pour démontrer l'efficacité de la dDIP *in vivo* chez la bactérie; les plugs d'agarose. En immobilisant les cellules dans une matrice d'agarose à faible température de fusion (*low melting point agarose*), il était possible de diminuer les dommages occasionnés par les manipulations avant le marquage, en plus de ne pas souffrir de l'agrégation naturelle des spermatides. La prochaine section décrit le développement de la technique grâce au modèle de cellules HeLa possédant la séquence I-SceI.

dDIP en plug : fixation, les plugs, la lyse et le marquage

Le modèle HeLa-ISceI

Afin de tester la capture des régions portant une cassure, nous avons créé un modèle de cassure bicaténaire inductible *in vivo* grâce à l'insertion de la séquence reconnue par la méganucléase I-SceI dans le génome de cellules HeLa. Grâce à une collaboration avec le laboratoire de Professeure Astrid M. Engel (Tulane University, Louisiane, USA), nous avons utilisé le système de transposition *Sleeping Beauty*. Brièvement, selon les recommandations du fabricant, nous avons incubé les cellules HeLa avec l'agent de transfection GeneCellin (BioCellChallenge, Toulon, France) en présence de deux plasmides, soit un premier comportant la séquence à transposer contenant la séquence de reconnaissance de I-SceI et un gène de résistance à la blasticidine (et des séquences de reconnaissance préférentielle de la nucléase provenant de LINE1 (L1), voir plus bas) et un deuxième plasmide exprimant la transposase *Sleeping Beauty*. Après quelques jours de sélection avec la blasticidine (Invitrogen, Ontario, Canada), nous avons procédé à une dilution limite pour déposer une seule cellule par puits par cytométrie de flux (BD FACSAria III, BD Biosciences, California, USA) grâce à une collaboration avec le laboratoire de Professeur Martin Richter (Université de Sherbrooke). Après quelques semaines d'incubation, certains clones ont été choisis pour leur croissance et leur apparence saine. L'ADN a été extrait et la présence de l'insert contenant la séquence I-SceI a été vérifiée et quantifiée par PCR en temps réel (qPCR). Il a aussi été possible de faire quelques tests d'enrichissement de cette région en effectuant une digestion *in vitro* avec la méganucléase I-SceI disponible commercialement (Fermentas, maintenant Thermo Fisher Scientific, inc., Massachussetts, USA) en utilisant le plasmide portant l'insert ou l'ADN génomique de cellules HeLa-ISceI. Utilisant ce modèle cellulaire, Mélina Arguin pilote actuellement un projet, en collaboration avec le labo de Professeure Engel, qui a pour but de cartographier par dDIP les séquences préférentielles d'insertion du retrotransposon L1; par une approche similaire à la méganucléase I-SceI, elle transfecte un plasmide exprimant l'endonucléase du retrotransposon pour établir par

dDIP les séquences visées par celle-ci. Nous avons généré deux lignées clonales dont l'insert comportait la séquence de reconnaissance de I-SceI et aussi 3 ou 5 sites préférentiels pour l'endonucléase, servant ainsi de témoin positif pour cette expérience.

La fixation

Pour cette étape, nous avons opté pour une fixation à l'éthanol froid. Cette méthode nous permet de fixer les cellules en les perméabilisant sans avoir les désavantages des liens covalents (protéines-ADN) créés par des fixateurs à base d'aldéhydes qui diminuent grandement le marquage de la TdT. Nous avons testé plusieurs types d'alcool et c'est l'ajout lent et délicat d'éthanol 100% froid (-20°C) jusqu'à une concentration finale de 70% dans un tampon supplémenté en EDTA qui s'est avéré le plus efficace, le plus compatible avec la lyse et le marquage de la TdT, et surtout entrainant le moins de dommages dus à la fixation. Les détails de la fixation sont expliqués à l'Annexe 4.

Les *plugs* d'agarose

Une fois les cellules fixées, elles ont été incluses dans l'agarose à basse température de fusion (agarose LMP). Brièvement, nous avons préparé une solution de 2% d'agarose LMP dans du PBS stérile en chauffant la solution à 65°C sur un bloc chauffant. Après la fusion de l'agarose, la solution d'agarose 2% était maintenue à 40-45°C jusqu'à son utilisation. Les cellules fixées (préalablement réchauffée à 37°C pour s'assurer que l'agarose ne fige trop vite) étaient alors mélangées avec un ratio 1 :1 avec l'agarose, vortexées et rapidement appliquées au moule de plugs (environ 80µl par puits). Ces plugs de 1% agarose LMP étaient alors laissés à température pièce jusqu'à ce qu'ils gélifient et transférés dans des tubes de 2 ml. Plusieurs concentrations d'agarose ont été testées (0,5% à 3%) et les plugs de 1% agarose offraient le meilleur

compromis entre l'accessibilité et la conservation de l'intégrité des plugs au cours de l'expérience (lors des incubations et lavages).

La lyse

S'inspirant de protocoles d'électrophorèse en champ pulsé (PGFE), nous avons cherché à adapter les tampons pour effectuer une lyse cellulaire efficace autant pour les cellules HeLa que pour les spermatides. Au moment la mise au point de ce protocole, une autre étudiante dans le laboratoire Boissonneault, Marie-Chantal Grégoire, venait de mettre au point un protocole de COMET (single cell electrophoresis) pour les cellules germinales mâles; nous avons donc mis en commun les informations de ces deux protocoles pour constituer le meilleur tampon de lyse universel tant pour les cellules HeLa que pour les spermatozoïdes. La lyse a été testée par un test d'accessibilité à la Dnase I (un exemple est présenté à la Figure A3-3). Les meilleures conditions obtenues sont une incubation à 55°C pendant 16 heures dans une solution de lyse contenant 2,5 M de NaCl, 10 mM Tris-HCl, 100 mM EDTA, 10 mM DTT, 0,01 mg/ml de protéinase K et 1% Triton X-100 (ajusté à un pH 8).

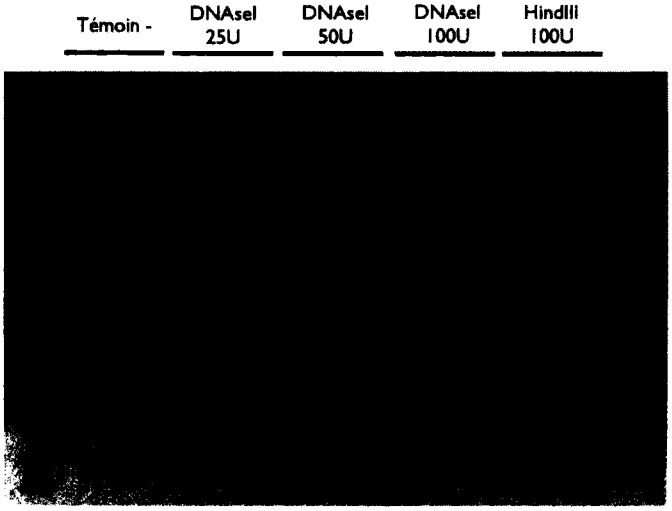

Figure A3-3. Test d'accessibilité à la Dnase I et HindIII suite à l'inclusion des cellules HeLa-ISceI en plug d'agarose LMP 1% et lyse de 16h à 55°C.
Après digestion, les plugs ont été insérés dans les puits d'un gel d'agarose 0,8%. Lorsqu'intact, l'ADN génomique est trop grand pour migrer hors des plugs, ce qui n'est pas le cas après une digestion avec la Dnase I ou HindIII.

Enrichissement dDIP avec cellules HeLa-ISceI transfectée

Afin de s'assurer de fonctionnement de notre nouveau système de cassure bicaténaire inductible, nous avons effectué avec succès un test utilisant le plasmide digéré *in vitro* avec la méganucléase I-SceI avec une approche similaire décrite au Chapitre 2 (voir Figure A3-4). Malheureusement dû à la nature même de la méganucléase I-SceI, l'enrichissement était minime lorsque nous effectuions ce même test avec le plasmide ou l'ADN génomique de HeLa-ISceI avec une digestion I-SceI une fois l'ADN inclus dans les plugs, car les méganucléases restent liées à leur séquence de reconnaissance contrairement à une endonucléase normale. Il aurait fallu utiliser des conditions incompatibles avec les plugs d'agarose pour la décrocher définitivement.

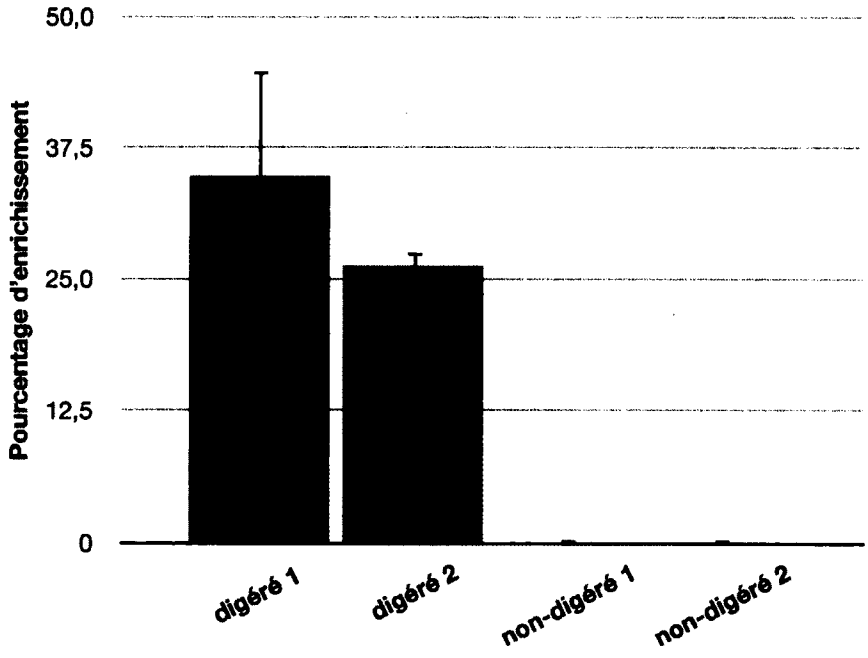

Figure A3-4. Enrichissement par dDIP avec un plasmide, portant la séquence de reconnaissance I-SceI, digéré *in vitro* par I-SceI.
Comparaison de l'enrichissement en duplicata mesuré par qPCR du plasmide SB I-SceI digéré ou non-digéré *in vitro* par I-SceI. Après le marquage, l'ADN a été digéré par PciI pour fragmenter le plasmide en fragments de taille idéale pour l'immunoprécipitation.

Suite à ces tests de dDIP en solution, nous avons voulu optimiser le marquage TdT en plug. Pour ce faire, en se basant sur nos résultats précédents avec la bactérie,

nous avons testé plusieurs conditions (voir Figure A3-5). Le meilleur marquage a été obtenu avec une incubation à 4°C pendant 16h, permettant à l'enzyme et les nucléotides de pénétrer dans l'agarose, suivi d'une incubation de 2h à 37°C.

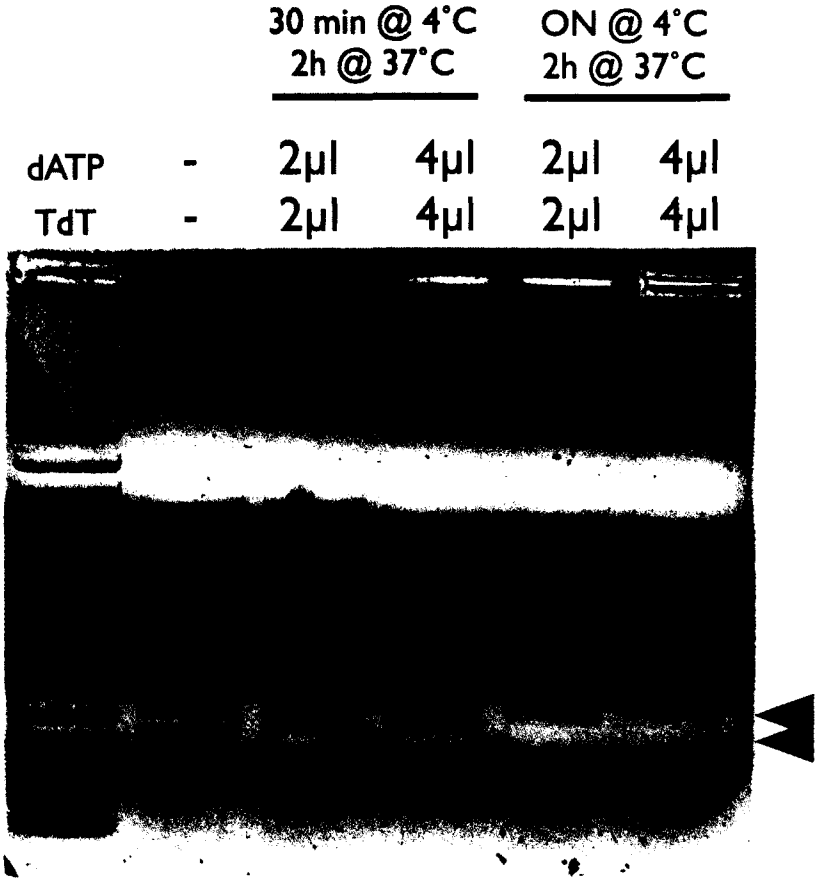

Figure A3-5. Test de marquage du plasmide SB I-SceI par la TdT en plug d'agarose LMP 1% avec du dATP
L'ADN du plasmide SB I-SceI, digéré par I-SceI *in vitro*, a été inclus dans l'agarose LMP, puis incubé en présence du tampon de marquage contenant de la TdT et du dATP. Après l'incubation, l'ADN a été extrait par une digestion β-agarase suivi d'une précipitation à l'éthanol. L'ADN a été ensuite digéré par l'endonucléase PciI pour créer des fragments de taille optimale pour suivre l'addition de dATP au site de cassure créée par I-SceI (les deux bandes du bas sur le gel indiquées par des flèches).

Pour simuler une expérience complète de capture de séquences endommagées, nous avons transfecté les cellules HeLa-ISceI avec un plasmide exprimant la méganucléase I-SceI pendant 24h, fixé les cellules avec l'éthanol froid, inclus celles-ci dans l'agarose, effectué la lyse et le marquage (cette fois-ci avec du dATP-biotine et du dATP), tel que décrit plus haut. La nébulisation a été utilisée pour fragmenter l'ADN de

façon aléatoire créant des fragments de tailles idéales pour l'immunoprécipitation, c'est-à-dire des fragments entre 200 et 1000 pb (voir la Figure A3-6). Nous avons aussi utilisé des cellules non-transfectées comme témoin négatif. Cette expérience a été répétée à plusieurs reprises par d'autres membres du laboratoire Boissonneault avec plus de témoins (transfection avec le plasmide pcDNA3, par exemple) avec autant de succès.

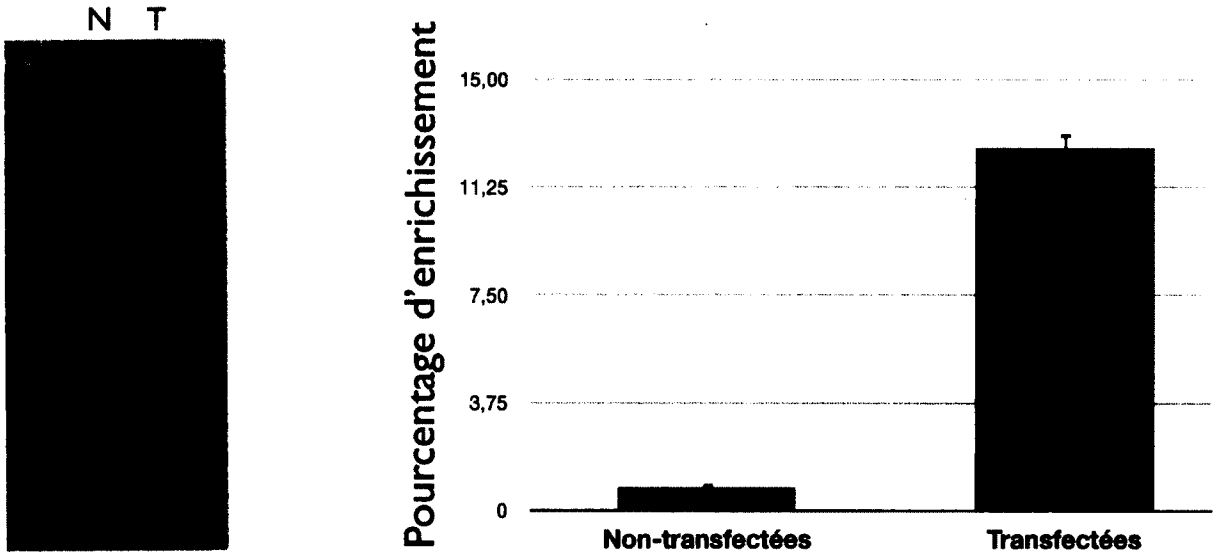

Figure A3-6. Enrichissement par dDIP de la séquence de l'insert chez des cellules HeLa-ISceI transfectées ou non avec le plasmide exprimant la méganuléase I-SceI.
À gauche, gel d'électrophorèse de l'ADN extrait des plugs et nébulisé montrant un patron de fragments entre 300 et 1000 pb pour de l'ADN de cellules non-tranfectées (N) et transfectées (T) avec le plasmide exprimant la méganucléase I-SceI. À droite, enrichissement de l'insert (région flaquante de la séquence de reconnaissance de I-SceI) par rapport à l'ADN de départ avant immunoprecipitation, mesuré par qPCR.

Une méthode universelle pour cartographier les cassures en plug d'agarose

Nous avons donc développé une approche universelle pour marquer les cassures de cellules d'eucaryotes supérieurs en diminuant la possibilité de créer des cassures non-spécifiques avant le marquage de la TdT. Cette approche est compatible avec les spermatides allongeantes grâce à un tampon de lyse optimisé et une

immobilisation dans l'agarose. Bien que cette approche semble très bien fonctionner avec notre modèle cellulaire HeLa-ISceI, celle-ci comporte quelques limitations. Premièrement, l'inclusion en agarose permet aux enzymes d'atteindre l'ADN des cellules immobilisées, mais il est aussi possible que les petits fragments d'ADN provenant des régions très endommagées (plus petits que 500 pb) diffusent hors de la matrice d'agarose et soient perdus pendant les incubations et les lavages. De plus, l'extraction de l'ADN des plugs d'agarose par une digestion avec la β-agarase suivie d'une précipitation à l'éthanol n'est pas toujours très efficace et certaines pertes importantes (10-60%) ont déjà été observées. Il est essentiel d'ajouter un véhicule inerte, tel que le glycogène, et d'effectuer de longues incubations au congélateur (16-24 heures à -20°C) avant de centrifuger. Dans l'ensemble, cette approche semble adéquate pour entreprendre la cartographie des cassures bicaténaires transitoires des spermatides allongeantes. Par contre, nous n'avions pas encore de techniques nous permettant de purifier une quantité suffisante de cellules pour effectuer cette expérience. Il était toujours possible d'utiliser le STAPUT pour purifier les cellules haploïdes en grande quantité, mais les cassures dues à la transcription très active des spermatides rondes allaient peut-être noyer celles du remodelage de la chromatine.

Purification des spermatides par FACS

Il fallait donc trouver une meilleure façon de purifier les spermatides allongeantes pour obtenir une distribution claire des cassures bicaténaires de ces cellules. La première tentative avec les squashs suggérait fortement que de décomposer *a posteriori* l'origine des cassures provenant d'événements cellulaires différents était très difficile, voire impossible. À l'époque, on mesurait l'intensité de différents marquages (TUNEL, immunofluorescence de l'histone H4 hyperacétylé et du variant d'histone γH2AFX, coloration d'ADN) sur des préparation squash de stades spécifiques en utilisant un cytomètre sur lame iCys (Compucyte, Massachussetts, USA). Grâce à un algorithme développé avec le responsable de l'unité d'imagerie, Éric Bouchard, utilisant l'intensité de la coloration de l'ADN et la surface du noyau, on

pouvait classer les cellules germinales automatiquement. Par contre, les spermatides allongeantes se retrouvaient constamment à l'extérieur des barèmes de sélection, car la coloration de leurs noyaux était trop intense, phénomène que nous avions déjà remarqué sur les coupes de tissus testiculaires et sur les préparations squash. Avec cette évidence, nous sommes revenus sur nos résultats de purification par FACS (début de doctorat) pour réaliser que la stratégie de sélection était inappropriée.

En utilisant la taille et granulosité des cellules ainsi que la coloration de l'ADN pour établir des populations cellulaires, nous cherchions à l'époque les spermatides allongeantes dans la population qui représentait une coloration d'ADN « haploïde », sans jamais les trouver en grand nombre. Nous avions même testé sans succès un colorant de mitochondries, le 10-nonyl acridine orange, pour tenter de les séparer plus facilement, car les spermatides possèdent proportionnellement plus de mitochondries. Or, nous cherchions au mauvais endroit; les spermatides allongeantes démontrent une coloration de l'ADN qui surpasse largement leur ploïdie. Avec cette nouvelle façon d'analyser les populations cellulaires en cytométrie de flux et l'utilisation d'un colorant perméable utilisé principalement pour les spermatozoïdes (SYBR-14), nous avons mis au point une stratégie de sélection au FACS, avec la collaboration du laboratoire de Professeur Martin Richter, qui permet d'isoler avec une très grande pureté (95-100%) plusieurs types cellulaires dont les spermatides allongeantes. Celles-ci se retrouvent en intensité de coloration de l'ADN avec les cellules tétraploïdes, soit quatre fois plus « colorées » que leur ploïdie théorique. Ceci peut être expliqué par l'état particulier de leur chromatine, dont l'éjection des histones (remplacées par les protéines de transition puis finalement par les protamines) donnerait au colorant un accès plus grand à l'ADN.

Suite à la première mise en place du protocole de séparation des cellules germinales, un effort d'équipe a été entrepris pour optimiser cette approche augmentant ainsi le nombre de cellules triées par population. Un article méthodologique a été écrit et peut être consulté en Annexe 4.

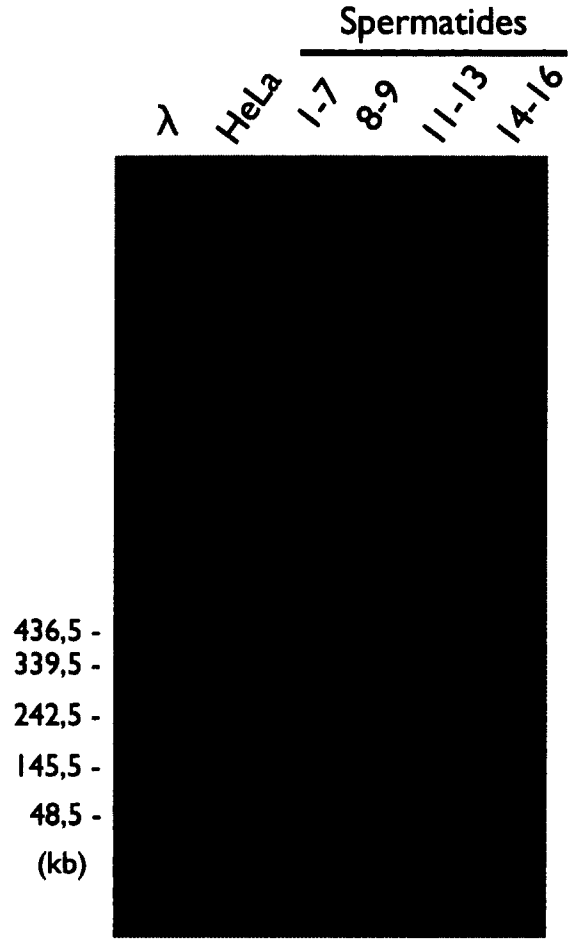

Figure A3-7. Migration PFGE de l'ADN de cellules germinales mâles triées par FACS.
Les cellules germinales ont été sélectionnées par leur patron tubulaire en transilluminance (voir Annexe 4, box 1), fixées et triées par FACS. Les cellules triées ont été incluses dans des plugs d'agarose LMP 1% (400 000 cellules par plugs) et lysées tel que décrit plus haut. Les plugs ont été insérés dans un gel d'agarose 1,5% et la migration de 6 volts en alternance de 60-120s (*switch time*) a été faite sur un système CHEF DR II (Bio-Rad) avec un tampon de TBE 0,5X pendant 30h.

Grâce à cette nouvelle méthode d'isolation des populations germinales, nous avons ajouté une évidence supplémentaire démontrant la présence de cassures bicaténaires transitoires chez les spermatides allongeantes (étapes 9-13) par une migration par champs pulsé par électrophorèse sur gel (PFGE) des spermatides triées par FACS (voir Figure A3-7).

dDIP sur cellules triées par FACS : quantité requise pour le séquençage de deuxième génération

Le protocole

Dès la première version fonctionnelle du tri de cellules germinales par cytométrie avec le SYBR-14, nous avons tenté la dDIP avec l'équivalent de trois jours de tri (voir la Figure A3-8 pour le plan d'expérience). Cinq populations ont été recueillies, soit les spermatocytes zygotènes/début pachytènes (représentant la méiose), les spermatides rondes (étapes 1-9, avant le remodelage de la chromatine et initiation du remodelage chromatinien), les spermatides allongeantes (étapes 10-13, en remodelage de la chromatine), les spermatides allongées (étapes 14-16, après le remodelage de la chromatine) et les spermatozoïdes (provenant des épididymes et des *vas deferens*). Les résultats du tri sont répertoriés dans le Tableau A3-1. La pureté de chaque population a été vérifiée par microscopie en épifluorescence grâce à une coloration de l'ADN par le DAPI. Les étapes d'inclusion dans l'agarose (maximum de 1 000 000 cellules par plug) et la lyse ont été effectuées telles que mentionné à la section précédente.

Tableau A3-1. Cellules triées par FACS et leur pureté pour la deuxième tentative de dDIP sur les cellules germinales mâles.

Populations	**Jour 1** nombre de cellules triées (% pureté)	**Jour 2** nombre de cellules triées (% pureté)	**Jour 3** nombre de cellules triées (% pureté)
Spermatocytes méiotiques	250 000 (100%)	655 000 (100%)	509 000 (100%)
Spermatides rondes	800 000 (100%)	4 290 000 (100%)	1 775 000 (100%)
Spermatides allongeantes	250 000 (95%)	1 090 000 (95%)	451 000 (98%)
Spermatides allongées	200 000 (100%)	219 000 (99%)	193 000 (90%)
Spermatozoïdes	400 000 (92%)	3 328 000 (60%)	1 604 000 (80%)

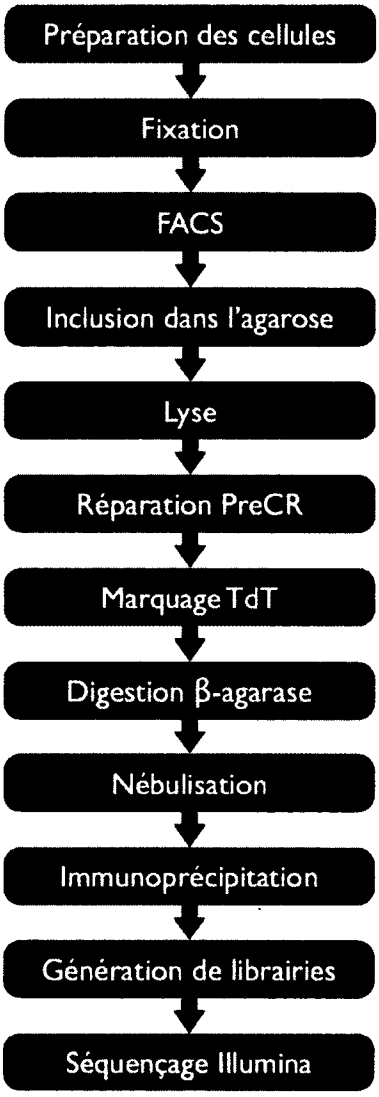

Figure A3-8. Plan d'expérience dDIP à partir de cellules germinales triées par FACS.

Par contre, après la lyse, nous avons ajouté une étape de réparation avec le mélange d'enzymes PreCR (New England Biolabs, Massachusetts, USA) avec une incubation pendant 16h à 4°C suivi de 3h à 37°C. Ce mélange d'enzymes de réparation a la capacité de réparer plusieurs types de dommages à l'exception des cassures bicaténaires (UV, oxydatifs, sites abasiques) mais c'est surtout pour sa capacité à réparer les cassures monocaténaires et de retirer les extrémités 3' bloquées que nous l'avons utilisé; d'une part, il pouvait retirer le bruit de fond dû aux cassures

monocaténaires généralement associées à la transcription et de l'autre, il libérait les extrémités 3' d'ADN qui pouvaient être bloquées par la topoisomérase IIβ catalytiquement immobilisée (du moins les restes de l'enzyme digérée par la protéinase K provenant de la lyse), une hypothèse que nous avons suggérée pour expliquer la présence de cassures prolongées chez les spermatides allongeantes (voir Chapitre 1).

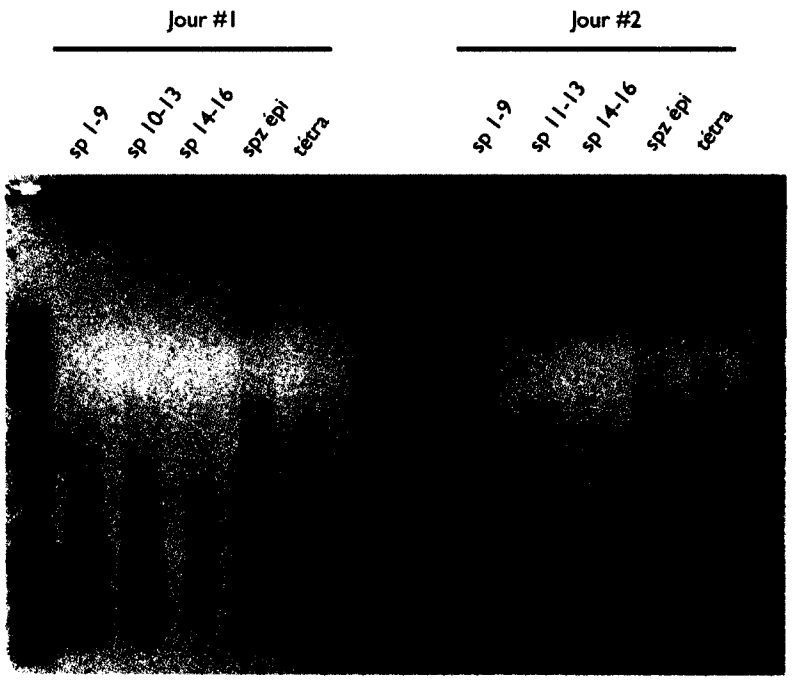

Figure A3-9. Électrophorèse de l'ADN marqué nébulisé provenant de cellules germinales triées au FACS (jour 1 et jour 2).

Pour le marquage TdT, les plugs ont d'abord incubé dans le tampon de TdT sans les nucléotides et la TdT pour équilibrer les plugs avec le milieu réactionnel. Puis, il a été remplacé par 200µl de tampon de la TdT supplémenté avec 2 µl de dATP 10 mM, de 5 µl de Biotine-dATP 0,4 mM et 800 unités de TdT (par plug). Les tubes ont été placés à 4°C pendant 16h et le lendemain, incubé à 37°C pendant 3h sur un bloc chauffant. Une fois le marquage terminé, les plugs ont été lavés trois fois avec 1 ml de TE froid pendant 30 min. L'ADN marqué a été extrait des plugs par une digestion β-

agarase selon le protocole court du fabriquant (New England Biolabs). Une fois l'agarose digéré, 800 µl d'une solution 50% glycérol-TE ont été ajoutés à chaque tube. Le contenu de chaque tube a été alors transféré dans un nébulisateur à usage unique (Life Sciences), et nébulisé avec de l'azote à une pression de 32 psi pendant 6 minutes. Le volume restant a été précipité à l'éthanol utilisant 10% LiCl 5M et 1µl de glycogène (20mg/ml). La précipitation a été effectuée à -20°C pendant 1h avant que l'ADN soit culoté par centrifugation.

Une fois resuspendu dans du TE, l'ADN marqué a été évalué sur gel (5-10% du volume sur gel) pour vérifier l'efficacité de la nébulisation (voir Figure A3-9). L'immunoprécipitation a été effectuée telle que décrite dans le Chapitre 2 avec la distinction que l'élution du complexe d'immunocapture a été faite en incubant les billes avec du TE avec 1% de SDS pendant 20 minutes à 65°C pour préserver l'intégrité et l'hybridation des brins d'ADN. L'ADN a ensuite été purifié en utilisant le kit Qiagen Minelute PCR cleanup pour retirer les traces d'anticorps dénaturés et de SDS.

La génération des librairies de séquençage

Pour un résumé du fonctionnement du séquençage de type Solexa (Illumina) et la génération de librairies de séquençage, veuillez consulter ce petit vidéo de 3 minutes : https://www.youtube.com/watch?v=l99aKKHcxC4.

Pour la génération des librairies de séquençage, nous avons opté pour le kit commercialement vendu par Illumina. Par contre, il fallait jumeler deux protocoles, soit la génération de librairies de type ChIP-seq (séquençage d'immunoprécipitation de chromatine), se rapprochant le plus de la dDIP, et le protocole de librairies « paired-end » indexées, afin de séquencer chacune des extrémités (*paired-end*) et de séquencer plusieurs librairies à la fois sur une même piste (indexation). Le séquençage de type « *paired-end* » est essentiel car il nous permet de déterminer le type de cassure, monocaténaire ou bicaténaire, grâce à l'identification liée des deux extrémités et un

patron d'enrichissement spécifique (voir la Figure A3-10). Le séquençage de type « *paired-end* » est régulièrement utilisé pour le séquençage de génome sans référence précise requiérant par conséquent un assemblage *de novo* ainsi que pour la transcriptomique (RNA-Seq) permettant une meilleure reconstitution des transcrits épissés ou encore pour identifier précisément les frontières des nucléosomes (MNase-Seq), mais il est rarement utilisé pour le ChIP-seq visant des facteurs de transcriptions car le gain serait minime puisque dans ce cas la résolution est surtout limitée par la longueur des fragments obtenus à l'étape de fragmentation de l'ADN (e.g. sonication, nébulisation). Comme nous partons avec la prémisse que les cassures bicaténaires transitoires des spermatides allongeantes devraient se répartir sur tout le génome puisque le remodelage de la chromatine touche l'ensemble du celui-ci, le séquençage « *paired-end* » nous assure un alignement plus précis (et moins de pertes durant l'alignement) dans les régions répétitives constituant près de 50% du génome de la souris et de l'humain.

Durant mon stage à Wayne State University, j'avais mis au point un protocole de génération de librairies de séquençage *paired-end* indexées à partir d'ADN enrichi par dDIP et je l'avais utilisé avec succès lors du premier séquençage à partir de l'ADN marqué extrait des préparations squash. Par contre, Illumina, ayant récemment fait l'acquisition de l'entreprise Epicentre, avait commencé à changer la composition enzymatique de leur kit, qui provenait anciennement de New England Biolabs. Suite à ces changements, nous n'avions pas réussi à générer les librairies de séquençage ni à partir du kit vendu par Illumina ni par celui vendu par New England biolabs. Même en testant le protocole original de chacun des kits avec de l'ADN génomique nébulisé, nous arrivions à peine à obtenir des librairies de séquençage satisfaisantes, et ce malgré de nombreux échanges avec l'assistance technique de chacune des deux entreprises. Ce faisant, nous avons gaspillé une grande partie de l'immunoprécipitat préparé à partir du tri de 3 jours.

Par contre, grâce à une collaboration avec Professeur Sébastien Rodrigue, nouvellement recruté dans le Département de Biologie (Université de Sherbrooke),

nous avons pu générer des librairies de séquençage utilisant une approche différente des kits commercialement disponibles(Rodrigue et al., 2010). Le séquençage s'est effectué au centre de génomique du Massachussetts Institute of technology (MIT). Accompagnant les librairies des cellules germinales mâles, deux autres librairies effectuées à partir d'ADN de cellules sénescentes enrichi par dDIP (projet piloté par Marie-Chantal Grégoire) ont aussi été séquencées. Malheureusement pour une deuxième fois en autant de tentatives, le séquençage n'a pas généré les résultats attendus, non pas cette fois-ci à cause de la qualité, mais à cause d'une suramplification PCR (représentation massive des mêmes lectures) lors de la génération des librairies due à trop peu d'ADN de départ. Malgré les 5-10 ng d'ADN d'immunoprécipitat restant après toutes les tentatives infructueuses avec les kits commerciaux, cette quantité s'est avérée trop petite pour obtenir des résultats valides. Par contre, les librairies représentant les cassures durant la sénescence cellulaire ont généré une distribution équilibrée avec peu de suramplification et sont présentement en processus de validation par qPCR.

dDIP avec cellules triées par FACS, revue et améliorée

Nous avons entrepris un nouveau tri de 4 jours avec le protocole amélioré de purification de cellules germinales mâles établi (décrit à l'Annexe 4) dans le but de recommencer l'expérience avec plus de matériel de départ. Grâce à ces améliorations, nous avons obtenus plus de 5 fois plus de cellules triées qu'à notre première tentative. De plus, le protocole dDIP en plug a été davantage optimisé pour minimiser le bruit de fonds et l'efficacité de capture. Ainsi, nous croyons qu'en immunoprécipitant l'ensemble des quatre jours de tri (comparativement à une fraction de 3 jours de tri au second essai), nous obtiendrons assez d'ADN enrichi pour générer des librairies de séquençage riches indiquant les régions préférentielles pour les cassures de la méiose et du remodelage chromatinien des spermatides.

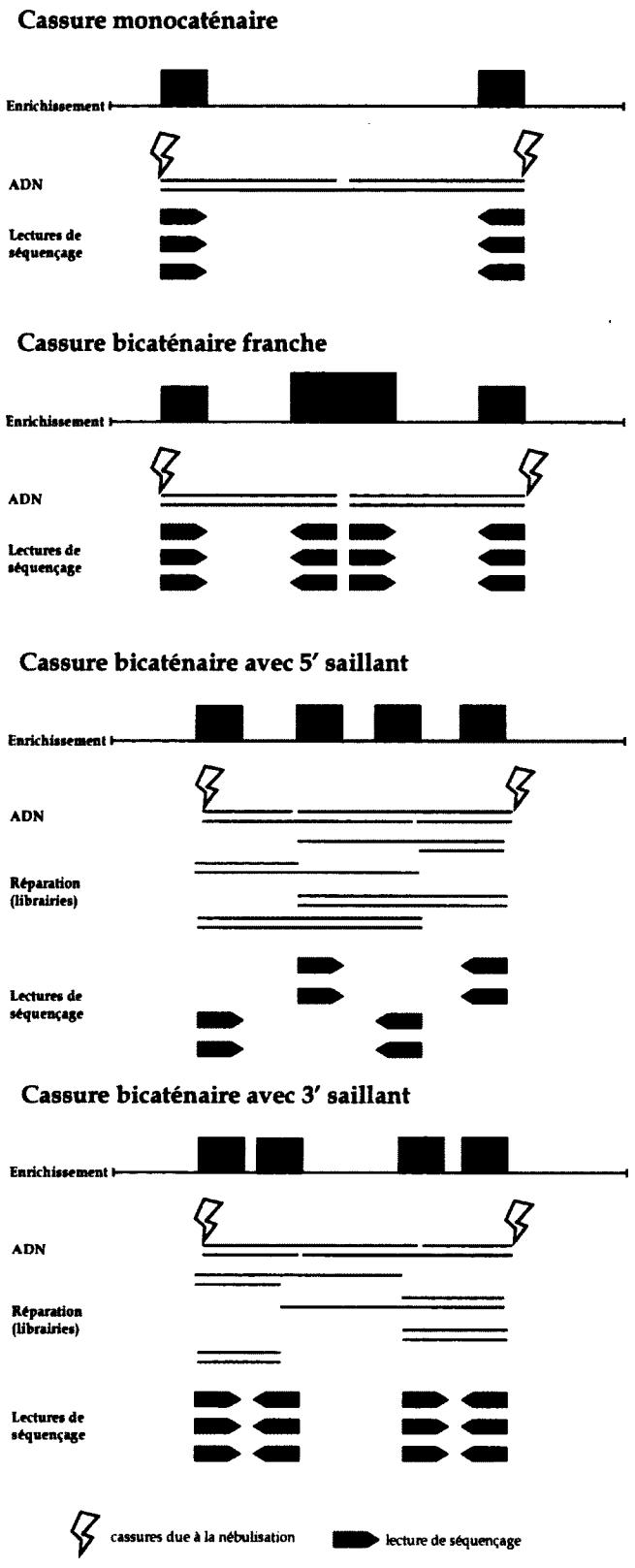

Figure A3-10. Patrons d'enrichissement théorique pour une cassure monocaténaire et bicaténaire suite au séquençage d'une librairie « *paired-end* » et d'un alignement génomique.

Adaptation pour les dommages UV et oxydatifs

Bien que le protocole original de dDIP ait été mis au point pour capturer seulement des cassures de l'ADN, nous avons longtemps pensé l'utiliser pour enrichir d'autres dommages à l'ADN, tels que les dommages causés par les radiations ultraviolettes (UV), les dommages oxydatifs ou les adduits liés à certains traitements oncologiques (l'agent anti-néoplasique cisplatine, par exemple). Afin d'adapter la dDIP à différents types de dommages, il fallait établir une façon de bloquer les extrémités (cassures) déjà présentes et d'identifier une ou plusieurs enzymes capables de transformer les dommages visés en cassures marquables (3'OH) sans que celles-ci altèrent le blocage préalable. Voici le résumé du développement de l'adaptation dDIP pour les dommages liés aux UV et les dommages oxydatifs.

Stratégie de blocage avec la terminal transférase et du ddATP

Nous avons utilisé une approche familière, soit le marquage avec la terminale transférase, qui offre un marquage efficace, rapide et souvent complet, afin que toute extrémité d'ADN non-spécifique (due à la transcription ou la réplication, par exemple) ne puisse diluer l'enrichissement spécifique d'un type de dommages à l'ADN. En incubant l'ADN à tester avec la terminale transférase et un didéoxynucléotides, il serait alors possible d'ajouter un nucléotide qui ne permettrait pas l'élongation de cette extrémité dû à l'absence d'un groupement hydroxyl en 3'. Nous avons opté pour le ddATP car l'adénosine est le substrat préféré de la TdT assurant le meilleur blocage possible.

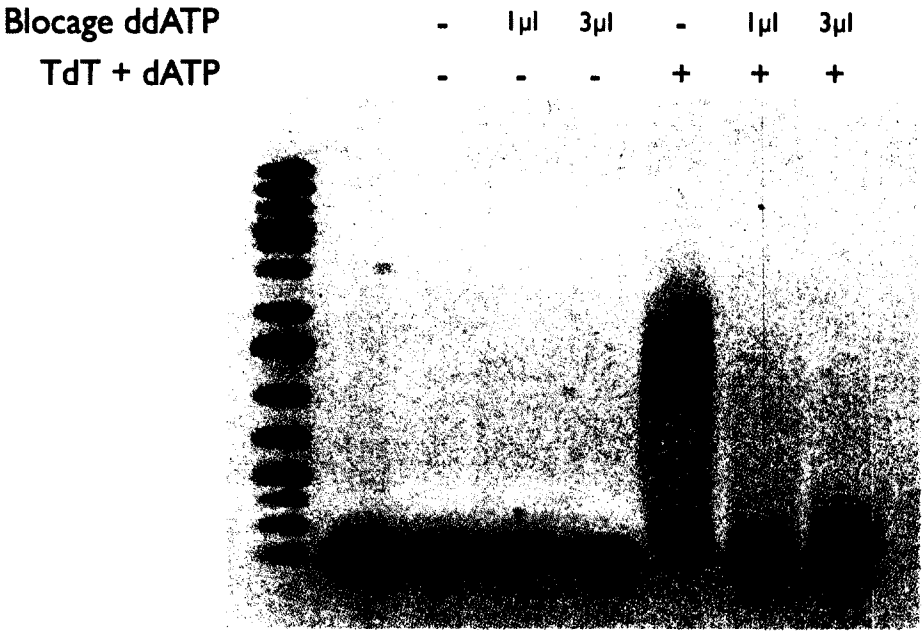

Figure A3-11. Test de blocage d'un produit de PCR d'environ 200 pb à l'aide de la TdT et ddATP.
Le produit de PCR a été tout d'abord incubé en présence de TdT sans nucléotide (-), 1µl et 3µl de ddATP 10mM. Après l'étape de blocage de 1h à 37°C, L'ADN a été purifié grâce à un kit Qiagen Minelute PCR cleanup et soumis à une deuxième incubation avec la TdT mais cette fois-ci avec 1µl de dATP pour vérifier l'efficacité de blocage de la première étape.

Utilisant un test *in vitro* à l'aide d'un produit de PCR d'environ 200 bp, nous avons démontré l'efficacité de ce type de blocage, tel que montré à la Figure A3-11. Afin de se rapprocher des conditions expérimentales correspondant à un dDIP, nous avons utilisé le modèle plasmidique pcDNA3 avec des cassures monocaténaires créées par l'enzyme Nt.BSPQI (New England Biolabs) tel que rapporté dans les données supplémentaires de l'article paru dans PLoS One. Comme la plupart des enzymes de réparation des dommages que nous visons créent généralement une cassure monocaténaire, il était donc approprié d'utiliser Nt.BSPQI, une endonucléase de type « *nicking enzyme*», pour simuler une activité similaire sur l'ADN en créant une cassure monocaténaire. Afin de mettre au défi notre étape de blocage, nous avons soniqué le plasmide pcDNA3 pour créer un nombre important de cassures non-spécifiques. Nous avons effectué une étape de blocage avec la TdT et du ddATP (1µl et 2µl de ddATP 10 mM), puis purifié l'ADN avec un kit commercial. L'ADN bloqué a été ensuite digéré par

l'enzyme Nt.BSPQI. Après l'inactivation de celle-ci, nous avons effectué un marquage dDIP avec un mélange de dATP et biotine-dATP, et immunoprécipité les fragments marqués. Pour évaluer l'efficacité du blocage, nous avons comparé les deux échantillons bloqués avec des témoins positif et négatif, qui ont été soniqués après le marquage dDIP plutôt qu'avant la digestion Nt.BSPQI et n'ont pas subi l'étape de blocage. L'enrichissement a été vérifié par la mesure de deux amplicons, soit l'amplicon 2700 (A) représentant un fragment endommagé spécifiquement par Nt.BSPQI (enrichissement important), et l'amplicon 4300 (D) correspondant à une région intouchée par Nt.BSPQI (enrichissement représentant le bruit de fond non-spécifique). Les résultats sont résumés à la Figure A3-12. Si le blocage n'avait pas été efficace, nous aurions enrichi les fragments mesurés par l'amplicon 4300 (D), alors qu'une très légère augmentation de l'enrichissement de cet amplicon est observée (0,61 ± 0,2 %et 0,52 ± 0,03% pour 2µl ddATP et 1µl ddATP vs. 0,18± 0,002% pour le témoin +). De plus, l'efficacité de l'enrichissement de l'amplicon 2700 (A) demeure inchangée démontrant que nous avons réussi à éliminer le ddATP surnuméraire de l'étape de blocage.

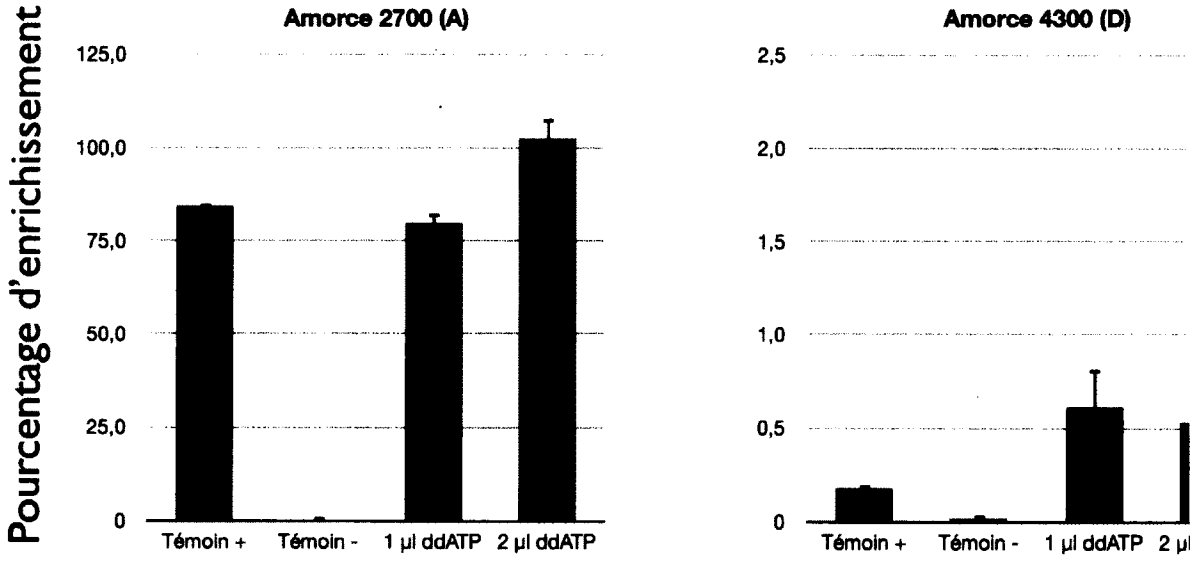

Figure A3-12. Évaluation de l'enrichissement spécifique par qPCR d'un fragment endommagé par l'endonucléase Nt.BSPQI à partir d'ADN plasmidique soniqué.

Conversion des dommages à l'ADN en cassure

Pour convertir les dommages en cassures, nous avons cherché une enzyme ou une série d'enzymes capables de générer une extrémité 3'OH afin de permettre un marquage par la TdT (voir Figure A3-13 pour notre stratégie finale). De façon surprenante, il a été très difficile de trouver une solution enzymatique simple à ce problème. Nous nous sommes d'abord attaqué à la conversion des dommages UV, car l'enzyme T4 endonucléase V était depuis très longtemps utilisée pour convertir les dimères de thymines (CPDs) en cassures. Par contre, l'extrémité laissée suite à son activité est plutôt instable et ne permet pas l'ajout de la TdT. Nous avons donc cherché une enzyme capable de polir ce type d'extrémité. En s'inspirant du mélange PreCR (New England Biolabs), nous avons identifié l'endonucléase IV (provenant de *E. coli*) comme candidate idéale pour cette tâche. Malheureusement celle-ci possède une petite activité exonucléase (non-décrite) qui est incompatible avec notre blocage avec du ddATP (voir Figure A3-14).

Finalement, nous avons identifié l'enzyme UVDE (*Ultraviolet Damage Endonuclease* provenant de la levure *S. pombe*, commercialement disponible chez Trevigen, Maryland, USA) ayant la capacité de reconnaître tous les dommages engendrés par le rayonnement UV (contrairement à la T4 endo V qui ne reconnaît que les CPDs) et qui convertit directement tous ces dommages en cassures monocaténaires avec des extrémités 3' hydroxyl. Ce projet s'est vite transformé en collaboration avec le laboratoire de Professeur Antonio Conconi qui étudie les dommages induits par les radiations UV chez la levure *S. cerevisiae*.

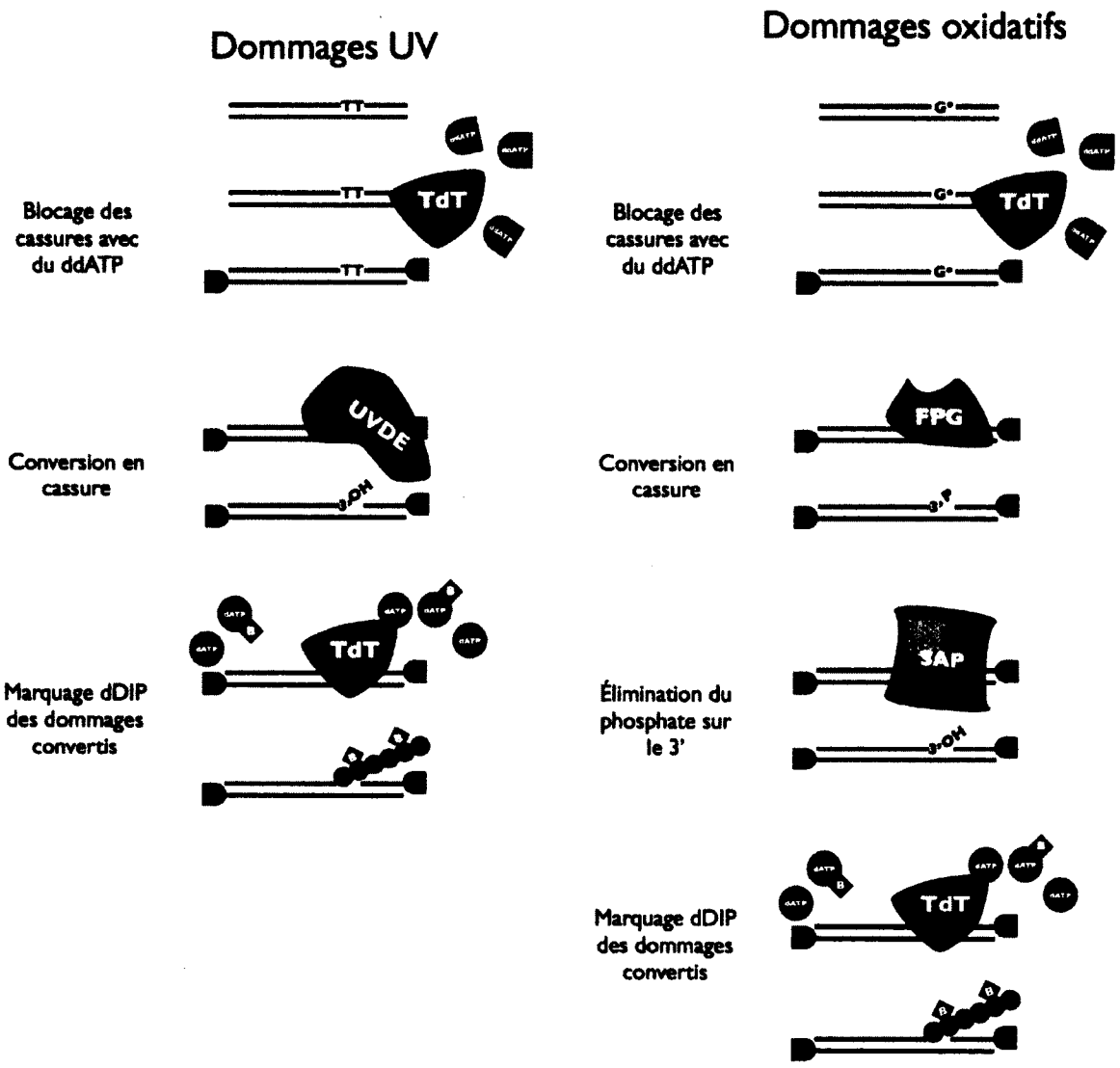

Figure A3-13. Stratégie de conversion des dommages UV et oxydatifs en cassures.

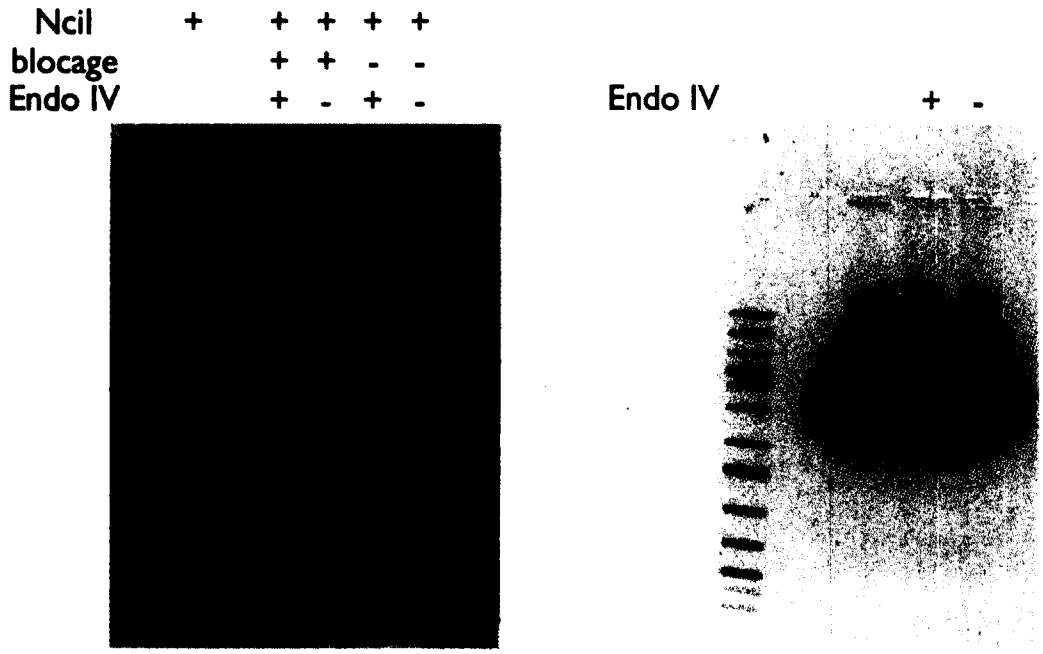

Figure A3-14. Test d'activité d'exonucléase et d'endonucléase de l'enzyme Endo IV.
À gauche, test d'activité d'exonucléase : le plasmide pcDNA a été digéré par NciI pour générer des fragments de tailles différentes, et bloqué avec la TdT et du ddATP. L'ADN a été par la suite incubé (ou pas) en présence de l'endo IV. Après une purification par colonne, l'ADN a été marqué par la TdT et du dATP pour vérifier l'intégrité du blocage. Le blocage est particulièrement affaibli suite à une incubation avec l'endo IV. À droite, test de l'activité endonucléase : le plasmide pcDNA3 a été mis en présence de l'endo IV pour vérifier la présence d'une activité endonucléase ou la présence de sites abasiques. Aucune activité

Depuis quelques mois, Annie D'Amours, professionnelle de recherche engagée pour mener à terme ce projet, a optimisé les étapes de blocage et de conversion adaptées au modèle cellulaire *S. cerevisiae*. Grâce à cette adaptation de la dDIP, surnommée UV-dDIP, nous sommes capables de suivre pour une première fois la réparation de tous les dommages UV simultanément et mettre en évidence la dynamique de réparation associée à chaque région génomique et ce, à l'échelle du génome au complet.

L'autre type de dommage d'ADN qui nous intéresse particulièrement est sans aucun doute les dommages oxydatifs. Souvent pointés du doigt pour nombreuses pathologies (sénescence et cancer, entre autres), les dommages oxydatifs représentent

une des causes possibles d'infertilité masculine qui, avec la fragmentation de l'ADN, s'établissent de plus en plus comme des biomarqueurs prédictifs de la fertilité. On ne connaît pas l'origine des espèces réactives d'oxygène (ROS) chez les spermatozoïdes, mais plusieurs études suggèrent des sources endogènes (activité mitochondriale) et exogènes comme la perte de protection antioxydante ou l'exposition à des composés xénobiotiques (pour une revue sur ce sujet, consultez cette publication(Aitken & de Iuliis, 2009)). Il y a donc un intérêt marqué dans le domaine de la biologie de la reproduction pour de nouveaux outils pouvant quantifier ou même cartographier les régions sensibles à ce type de dommage.

Nous explorons présentement la possibilité d'établir la distribution des dommages oxydatifs chez les spermatozoïdes murins grâce à deux modèles transgéniques développés par le laboratoire de Professeur Joël Drevet (Université Blaise Pascal, Clermont2, France). Les spermatozoïdes des souris GPX5$^{-/-}$ et GPX4$^{-/-}$ GPX5 $^{-/-}$ démontrent plus de dommages oxydatifs que ceux de la souris sauvage (Eléonore Chabory, 2009); les protéines GPX 4 et 5 (glutathione peroxidase) présentes au niveau des épididymes protègeraient les spermatozoïdes des attaques oxydatives. Pour ce faire, nous avons extrait l'ADN génomique des spermatozoïdes des souris transgéniques, GPX5$^{-/-}$ et GPX4$^{-/-}$ GPX5 $^{-/-}$, et sauvages. De plus, j'ai établi quelques preuves de concept validant le modèle suggéré à la Figure A3-13 en utilisant un produit de PCR dans lequel j'ai artificiellement incorporé des dommages oxydatifs grâce à l'addition de nucléotides 8-oxo-dGTP dans le pool de nucléotides utilisés pour l'amplification. Contrairement aux dommages UV où une seule enzyme créait une cassure marquable au site du dommage, la conversion des dommages oxydatifs requiert l'activité de deux enzymes, soit la FPG (formamidopyrimidine ADN glycosylase, New England Biolabs) qui reconnaît les 8-oxo-guanines et retire le nucléotide endommagé laissant une extrémité 3'phosphate, et la phosphatase alcaline de crevette (SAP, *shrimp alkaline phosphatase*, Fermentas) capable de convertir cette extrémité 3'P en 3'OH. Ce projet est maintenant piloté par Julien Massonneau, étudiant à la maîtrise au laboratoire Boissonneault. Nous espérons que dans un avenir proche, nous effectuerons la oxi-dDIP sur les échantillons fournis par Professeur Drevet et

établir la distribution des régions sensibles aux dommages oxydatifs chez les spermatozoïdes murins. De plus, il sera possible de quantifier le nombre exact de cassures par cellule avec cette approche oxi-dDIP en utilisant des nucléotides fluorescents, un test développé dans mon laboratoire d'accueil qui a déjà été utilisé avec succès avec des modèles de cassures *in vitro*.

Les adaptations de la dDIP pour d'autres dommages de l'ADN possèdent plusieurs avantages majeurs par rapport à sa méthodologie originale : l'étape de blocage retire un énorme bruit de fond, la conversion des dommages se fait sur de l'ADN extrait et non *in cellulo* ou en plug, et le marquage est aussi spécifique que l'activité enzymatique de conversion. Par contre, l'optimisation de ces adaptations demandera beaucoup d'efforts et une vérification constante de l'efficacité enzymatique à chaque étape. À moyen terme, ces approches seront une source inestimable de connaissances, qui mèneront à l'établissement de nouveaux biomarqueurs et de nouveaux traitements de l'infertilité et la mise en place de nouveaux outils plus performants et prédictifs.

Discussion et conclusion

La spermiogenèse, un programme de différenciation à caractère évolutif?

Étant un des articles les plus cités de ce journal pour l'année 2008, nos résultats publiés dans le journal BOR ont eu un impact significatif sur le domaine de la biologie de la reproduction. Ces observations ont été rapportées par la suite chez un grand nombre d'espèces, allant de l'algue à la sauterelle (Cabrero, Teruel, Carmona, & Camacho, 2007b; Rathke et al., 2007; Wojtczak et al., 2008), démontrant le caractère évolutif de ce programme particulier de la spermatogenèse. Plusieurs autres laboratoires ont aussi contribué à une meilleure caractérisation du processus de remodelage de la chromatine des spermatides, corroborant nos observations (Meyer-Ficca et al., 2011; 2009; Quénet, Mark, Govin, & van Dorsselear, 2009). Par contre, malgré de nombreux efforts techniques et expérimentaux de notre part, ce phénomène reste encore nébuleux. Bien que nous soupçonnons la topoisomérase IIβ d'être responsable des cassures transitoires d'ADN, le mécanisme par lequel une telle cassure serait visible par TUNEL ou même activerait la réponse aux dommages à l'ADN (*DNA damage response*) demeure inconnu. L'hypothèse la plus probable serait une activité de la topoisomérase IIβ modulée par les poly(ADP-ribosyl) polymérases (PARP) et la poly(ADP-ribose) glycohydrolase (PARG). La topoisomérase IIβ poly(ADP-ribosyl)ée serait capable de lier l'ADN et effectuer la coupure, mais ne pourrait pas effectuer la ligation, ce qui permettrait de libérer plus de supertours d'ADN que son activité habituelle. Une fois les ADP-riboses retirés par PARG, la topoisomérase IIβ pourrait effectuer la ligation (G. T. Zaalishvili, Tsetskhladze, Margiani, Gabriadze, & Zaalishvili, 2005). Ce modèle n'explique pas pourquoi la réponse au dommage à l'ADN est activé, visualisé par l'apparition de γH2AFX, ni la disponibilité des extrémités 3'OH (TUNEL ou ADN polymerase endogène).

Bien que γH2AFX pourrait avoir un rôle au niveau du remodelage de la chromatine plutôt que dans la signalisation des cassures (Fernandez-Capetillo et al., 2003; Szilard et al., 2010), cela n'explique pas comment la topoisomérase IIβ immobilisée et liée de façon covalente à l'extrémité 5' permettrait stochiométriquement un marquage par la TdT sur l'extrémité 3'. L'extrémité doit être disponible pour qu'un tel marquage soit possible, et donc si la topoisomérase IIβ est responsable de ces cassures, son activité est différente et modulée par un mécanisme inconnu. Il est aussi possible qu'après la coupure, la topoisomérase IIβ soit simplement retirée par un système de réparation, menant à l'activation de la signalisation de cassures bicaténaires. De plus, mes travaux et ceux d'autres étudiants dans le laboratoire suggèrent un rôle important de la jonction terminale non-homologue dépendant de DNApk (D-NHEJ) dans la réparation des cassures. Ce système de réparation est en effet connu pour induire certains types de mutation, généralement sous la forme de délétion, d'insertion ou de translocation.

Ce processus du programme normal du développement des spermatides, passant par des cassures bicaténaires transitoires à l'échelle d'un génome et une réparation propice à l'erreur, nous laisse croire que cette étape peut engendrer une plus grande diversité génétique. La spermiogenèse comporte donc une saveur évolutive insoupçonnée où chacun des spermatozoïdes pourraient porter de nombreuses mutations et contribuer au dynamisme du génome des êtres vivants utilisant ce procédé. En empruntant un concept d'écologie et reproduction évolutive développé dans les années 1970 (Losos, Ricklefs, & MacArthur, 2009), les spermatides représentent une version cellulaire de la stratégie de reproduction r (forte fécondité et faibles chances de survie) pour une espèce de type K (longue durée de vie, maturité sexuelle tardive, soins parentaux des jeunes). Non seulement les spermatides possèdent une diversité inhérente provenant de la ségrégation en 4 du bagage génétique du parent et son brassage lors de la méiose, mais individuellement de nouvelles modifications peuvent s'ajouter au cours de la spermiogenèse. Des études bien en dehors de la portée de cette thèse sont nécessaires pour vérifier la validité de cette hypothèse. Par contre, de nouvelles études de séquençage de familles chez

l'humain suggèrent un biais paternel important en ce qui concerne l'apparition de mutations *de novo* (Campbell et al., 2012; Conrad et al., 2011; Crow, 2000; Kong et al., 2012).

Or, il existe trois périodes qui pourraient engendrer ce biais mutagénique durant la spermatogenèse : la réplication des spermatogonies, la méiose des spermatocytes et le remodelage chromatinien des spermatides. Ce biais paternel peut être expliqué simplement par le plus grand nombre de division mitotique des spermatogonies par rapport à l'ovogenèse, démontré par un plus grand nombre de substitutions d'origine paternelle liées à la réplication de l'ADN. Avec l'avancement de l'âge paternel, il semblerait que ce biais de substitutions paternelles est amplifié (Kong et al., 2012). Bien qu'elle amène une diversité supplémentaire au niveau des haplotypes, la méiose ne semble pas être une source importante de mutations et les mutations qui peuvent en découler ne semblent pas avoir de biais parental car la méiose se produit chez les deux parents avec les mêmes composantes cellulaires. Il est donc possible qu'un déclin de l'efficacité des systèmes de réparation dû à l'âge de l'individu, pourrait favoriser des mutations supplémentaires après la méiose, plus spécifiquement pendant la spermiogenèse qui semble plus particulièrement vulnérable. Ultimement, une étude par dDIP des points de cassures les plus fréquents chez certains types de cellules germinales d'animaux à différents âges, telles que les spermatogonies, les spermatocytes en méioses, les spermatides en remodelage et les spermatozoïdes, complémentée d'un séquençage génomique de ceux-ci, procurerait une vue d'ensemble idéal pour bien comprendre la participation des différents types de réparation à travers la vie d'un individu. Par contre, une telle expérience demanderait énormement de ressources et une amélioration notée du séquençage de deuxième génération.

De plus, une étude a aussi démontré chez les rongeurs que d'autres types de mutation, les insertions et délétions (ou indels), comportent aussi une composante plus paternelle que maternelle (Makova, 2004). Or, les indels ne sont généralement pas associées avec la réplication de l'ADN mais beaucoup plus avec des systèmes de

réparation d'ADN, comme la NHEJ présente chez les spermatides (Ciccia & Elledge, 2010; Griffin & Thacker, 2004). Au cours de la spermatogenèse, la recombinaison homologue, un système de réparation fidèle, est le principal système de réparation présent, notamment chez les spermatogonies (réplication) et les spermatocytes (méioses). Le remodelage de la chromatine des spermatides, un processus absent durant l'ovogenèse, comporte les caractéristiques favorables à l'apparition d'indels : des cassures bicaténaires, absence de chromatide sœur ou chromosome homologue, aucun point de contrôle connu et la présence de la machinerie de NHEJ. La contribution du remodelage chromatinien des spermatides à la diversité génétique reste à être établie mais représente sans aucun doute un mécanisme insoupçonné de l'évolution des espèces.

Afin de mieux comprendre le mécanisme du remodelage de la chromatine des spermatides et évaluer son potentiel évolutif, l'élaboration d'animaux transgéniques dont la délétion de certains éléments serait conditionnelle et limitée à la spermiogenèse permettrait aux chercheurs d'explorer ce processus complexe avec plus de facilité. Comme plusieurs protéines impliquées lors de la spermiogenèse sont aussi impliquées plus tôt, une délétion permanente serait soit incompatible avec l'embryogenèse ou elle provoquerait l'arrêt prématuré de la progression d'étapes antérieures de la spermatogenèse, notamment durant les stades de spermatocytes pachytènes connus pour leurs points de contrôle menant à l'apoptose. Il serait donc essentiel de circonscrire la délétion à la phase haploïde pour mieux comprendre ce processus de remodelage de la chromatine des spermatides. Cette délétion pourrait être inductible, car nous pouvons suivre facilement la progression temporelle des différentes étapes de la spermiogenèse, ou déclenchée grâce à un promoteur spécifique à la spermiogenèse (celui des protamines, par exemple). Un tel système permettrait de mettre en évidence les acteurs principaux de ce processus complexe encore peu étudié.

La dDIP, un outil versatile

Pour mieux comprendre l'impact que pourrait avoir les cassures bicaténaires transitoires des spermatides, il fallait établir leur distribution à l'échelle du génome; une mutation due à une réparation inadéquate d'une cassure dans un gène devrait avoir plus d'impact pour la prochaine génération que dans une région intergénique. Or, devant l'inexistence d'une approche nous procurant cette information, nous avons développé la dDIP, dont la mise au point à partir de modèles *in vitro* et *in vivo* est parue dans le journal PLOSone en 2011. Pour son application à l'étude de l'instabilité génétique possiblement associée au remodelage chromatinien des spermatides, il a fallu d'abord élaborer une façon de marquer les cassures des spermatides sans compromettre l'intégrité de l'ADN, soit la dDIP en plug d'agarose, et l'établissement d'un nouveau protocole de purification de cellules germinales mâles par cytométrie en flux. Ainsi, la dDIP est en bonne voie de répondre à cette interrogation scientifique.

Limitations de l'approche

Nos tests avec les différents modèles nous ont permis d'établir quelques limitations de la dDIP pour étudier des processus complexes. Malgré la démonstration de l'efficacité avec le modèle cellulaire HeLa-ISceI, il est encore difficile d'établir avec certitude le seuil minimal de cassure à un endroit donné pour établir la validité d'un point chaud. En qPCR, nous arrivons à détecter avec des amorces spécifiques de très petits enrichissements (moins de 1%), mais ça ne sera peut-être pas suffisant lorsqu'appliqué à la technologie du séquençage de deuxième génération; en terme de nombre de fragments, l'enrichissement local dû à une cassure peut être inférieur à l'ensemble des fragments non-spécifiques ou qu'elle représente une cassure légitime dans chaque génome mais à différents endroits dans différentes cellules. De plus, il est aussi difficile de prévoir l'impact que pourrait avoir l'activité normale d'une cellule, et ses dommages endogènes, sur la cartographie d'un autre phénomène d'intérêt; les cassures endogènes pourraient masquer un phénomène induit ou pathologique. En ce qui concerne les spermatides allongeantes, ceci ne pose pas vraiment un problème car

la transcription chez ces cellules est presque inactive et les cassures dues au remodelage devraient constituer la majorité des dommages présents. Par contre, dans l'étude de la sénescence, il sera difficile de faire la distinction entre les dommages entrainés par la transcription par opposition aux cassures d'ADN qui s'accumulent lors de cette pathologie (Sedelnikova et al., 2004), à moins que celles-ci représentent un important point chaud.

Les adaptations de la dDIP à d'autres dommages ne souffrent pas de cette limitation car le bruit de fond cellulaire est éliminé par une étape de blocage. Seuls les dommages convertis seront cartographiés, amenant une distribution plus spécifique. Par contre, elle est dépendante de l'activité de conversion qui peut être non-spécifique ou inefficace. C'est pour cette raison qu'il est essentiel de vérifier chacune des étapes de ce processus avec des contrôles internes pour s'assurer du bon fonctionnement de cette approche.

Application à d'autres types de dommages et à d'autres domaines

Tel qu'il a été présenté au Chapitre 3, la dDIP peut être adaptée pour évaluer la distribution de différents types de dommages, autres que les cassures monocaténaires et bicaténaires. Ceci permet de mesurer à l'échelle d'un génome l'apparition et la réparation de dommages, et ainsi, de mieux comprendre les interactions des systèmes de réparation impliqués au site du dommage et l'influence de la chromatine sur leurs activités. Il sera donc possible d'avoir un point de vue global de la réparation sans négliger la résolution nucléotidique de la réparation. Ultimement, en choisissant les enzymes appropriées, il sera possible d'évaluer tout type de dommage à l'ADN, mais pour l'instant, nous avons identifié les enzymes nécessaires à la conversion des dommages causés par le rayonnement ultraviolet et les dommages oxydatifs.

La technique dDIP est un outil versatile car il peut être appliqué à tout domaine qui étudie l'intégrité génomique. D'ailleurs, au laboratoire, nous l'avons appliqué à l'étude

des cassures persistantes durant la sénescence et des régions préférentiellement visées par l'endonucléase de l'élément de retrotransposition LINE-1. Grâce à l'élaboration d'une méthodologie universelle en plugs, il est possible d'envisager la cartographie de plusieurs types cellulaires, et même de petits morceaux de tissus, comme des biopsies de tumeurs. Avec une évaluation des dommages présents, il est peut-être possible de déterminer les probabilités de carcinogenèse, en se basant sur l'apparition de mutations aux sites de cassures. Avec une approche similaire, il est aussi possible de déterminer si un traitement de chimiothérapie est efficace par l'intensité et la distribution des cassures dans des séquences d'ADN jugées essentielles à la survie cellulaire.

Le plein potentiel de la dDIP à la prochaine génération

À plusieurs reprises dans cette thèse, j'ai décrit la technique dDIP comme étant un outil qui permettait la cartographie des cassures (ou dommages convertis) à l'échelle d'un génome sans perdre la résolution nucléotidique, c'est-à-dire le site exact du dommage au nucléotide près. Cette précision nucléotidique est possible grâce à l'addition de plusieurs adénosines (dATP) à l'extrémité 3' de chaque dommage. À moins que le dommage se produise à proximité d'une adénosine (la base endommagée ou sa voisine en 3'), il est possible de déterminer au nucléotide près l'endroit de la cassure. Or, nous perdons cette information à cause de la génération de librairies de séquençage par ligation d'adaptateurs et amplification PCR avec l'approche par séquençage de deuxième génération, que ce soit sur la plateforme d'Illumina, de Roche 454, d'Ion Torrent ou de SOLiD. Au mieux, avec le séquençage de type *paired-end* sur la plateforme Illumina, il sera possible de cerner le site d'une cassure bicaténaire comme schématisé à la Figure A3-10. Nous avons tenté à plusieurs reprises d'utiliser une amorce spécifique (3'-NNTTTTTTTTTTTTTTT-adapteur Illumina-5') à l'extrémité marquée pour rendre le séquençage directionnel, c'est-à-dire séquencer spécifiquement le brin portant l'extension d'adénosines; en utilisant deux bases aléatoires (excluant l'adénosine) en 3' de l'amorce, on s'assurait que l'amorce

s'hybride au début de l'extension de la TdT, et l'oligo-(15)dT possédait la séquence d'adaptateur indexé des librairies d'Illumina, permettant l'amplification clonale sur la puce de séquençage. Hélas, la température d'hybridation de 15 thymines/adénosines est très basse (40-45°C) menant inévitablement à de l'amplification non-spécifique dans un génome possédant déjà plusieurs séquences riches en adénine.

Cependant, nous voyons apparaître de nouvelles technologies de séquençage, que l'on surnomme de troisième génération (*third generation sequencing*) qui nous permettront de profiter du plein potentiel de la dDIP. Ces technologies offrent le séquençage de molécules uniques, c'est-à-dire qu'il n'est pas nécessaire d'amplifier l'ADN d'intérêt avant l'analyse de celui-ci. Ainsi, il sera possible d'établir la distribution à l'échelle du génome tout en conservant le marquage de la TdT qui nous indiquera le site de cassure exact, et ce sans biais d'amplification PCR. Comme la génération actuelle de librairies de séquençage nécessite plusieurs étapes inefficaces (beaucoup de pertes), telles que la ligation d'adaptateurs et la sélection sur gel, il faut partir d'une grande quantité d'ADN pour obtenir un librairie représentative de l'échantillon original. Or, comme on analyse l'échantillon original sans ou avec très peu de modifications, il n'est pas nécessaire d'avoir une grande quantité de départ pour le séquençage de troisième génération. Bien que présentement il existe plusieurs systèmes en développement (Noblegen Biosciences, Stratos genomics, NABsys, Gnubio, un nanopore équipé d'un transistor développé par Roche 454 et IBM, entre autres), je me concentrerai sur deux systèmes, dont l'un est déjà disponible et l'autre le sera prochainement.

Pacific Biosciences a développé un appareil qui permet le séquençage dans de minuscules puits contenant une ADN polymérase. Le séquençage est possible grâce à l'utilisation de nucléotides dont le fluorochrome est placé sur la partie triphosphate plutôt que sur la base, contrairement aux technologies compétitrices. Lorsque la polymérase ajoute un nucléotide, celui-ci reste immobilisé pendant quelques millisecondes avant d'être polymérisé (pour une explication visuelle du principe, consultez http://www.youtube.com/watch?v=v8p4ph2MAvI). Cette méthode offre de

nombreux avantages : le séquençage d'une molécule unique, la génération d'un brin d'ADN entièrement naturel et le séquençage de longue séquence (de 2 à 10 kb). Par contre, cet appareil ne permet pas un très grand débit ce qui limite donc le nombre de brins pouvant être séquencé par heure et la qualité du séquençage est plutôt basse avec une moyenne de 85-90% au niveau de la précision de la détermination des bases (*base calling*). À moins d'une amélioration drastique de la capacité et de la précision, cet appareil demeura un outil idéal pour le séquençage de génome *de novo*.

Par contre, Oxford Nanopores Technologies (voir pour plus de détails, http://www.nanoporetech.com/technology/analytes-and-applications-dna-rna-proteins/dna-an-introduction-to-nanopore-sequencing) offre prochainement (sortie prévue en 2012) une alternative très intéressante utilisant des nanopores. En mesurant le changement d'impédance lorsque les nucléotides passent à travers d'un nanopore, il est possible de séquencer directement une molécule unique d'ADN. Appliqué à la dDIP, nous pourrions analyser uniquement les brins portant le marquage de la TdT et obtenir le site exact de la cassure ainsi que sa position sur le génome. La capacité des nanopores, bien que plus petite que les plateformes Illumina (MiSeq ou HiSeq), serait suffisante pour la dDIP et ce, à un coût comparable selon l'information disponible sur le site web de l'entreprise.

De nouvelles alternatives technologiques seront donc à surveiller pour améliorer l'efficacité et la spécificité de la dDIP et enfin permettre tirer le plein potentiel d'information, soit la région endommagée au nucléotide près, de ce nouvel outil.

Conclusion

Au cours de mes travaux de doctorat dans le laboratoire de Professeur Guylain Boissonneault, j'ai contribué à l'avancement des connaissances sur une étape critique de la spermatogenèse, soit le remodelage de la chromatine des spermatides, en démontrant la présence de la topoisomérase IIβ et d'une réponse active aux dommages à l'ADN lors des étapes 9 à 13 de la spermiogenèse. Afin de mieux comprendre le rôle des cassures bicaténaires transitoires observées à ces mêmes étapes, j'ai mis au point une méthode unique appelée dDIP (*damaged DNA immunoprecipitation*) qui permet l'enrichissement des séquences endommagées, leur cartographie à l'échelle d'un génome et la résolution au nucléotide près. À partir de nombreux modèles *in vitro* et *in vivo*, j'ai démontré la spécificité et l'efficacité de cette nouvelle approche pour la cartographie de cassures monocaténaires et bicaténaires. De plus, j'ai développé une approche douce et universelle en plug d'agarose pour effectuer la dDIP sur des cellules provenant d'eucaryotes supérieurs.

Dans le but de cartographier les cassures bicaténaires transitoires des spermatides allongeantes, j'ai aussi élaboré une nouvelle méthode de purification de cellules germinales mâles en cytométrie en flux en utilisant la coloration apparente d'ADN des différents types cellulaires (voir Annexe 4). Par contre, malgré deux essais infructueux, je n'ai pas réussi à atteindre mon objectif final, soit la cartographie des cassures transitoires des spermatides en remodelage chromatinien. Je suis cependant très confiant que les leçons apprises lors de ces deux tentatives m'ont poussé à améliorer cette méthodologie, que je considère maintenant mature, et qui devrait, avec la prise en charge de d'autres étudiants du laboratoire Boissonneault, permettre à très court terme d'atteindre mon dernier objectif. Grâce à deux collaborations, il sera aussi possible de cartographier les dommages causés par les rayonnements UV et les dommages oxydatifs en appliquant une stratégie de blocage et de conversion enzymatique des dommages en cassures marquables.

Le remodelage de la chromatine des spermatides allongeantes, un des plus grands bouleversements cellulaires observés dans le monde animal, demeure une étape relativement peu connue malgré plusieurs décennies d'études. Les limitations expérimentales (aucune culture possible à moyen terme, expériences *in vivo* difficiles, absence de modèles transgéniques conditionnels) représentent un défi constant. Il est donc extrêmement difficile d'effectuer des expériences quantitatives ou mécanistiques. Seules des études qualitatives, telles que présentées au Chapitre 1, sont généralement retrouvées dans la littérature. Évidemment, ce type d'étude est limité par la qualité des réactifs utilisés comme la spécificité des anticorps, qui doit être constamment contre-vérifiée. Le testicule est un organe passionnant qui abrite de nombreux processus uniques et complexes, qui rend une expérimentation simple (pour d'autres domaines d'étude) extrêmement complexe en ce qui concerne l'étude de la spermatogenèse. Et c'est pour cette raison que ma contribution au domaine de la biologie de la reproduction a surtout été méthodologique.

Dès mon arrivée dans le laboratoire, il s'est avéré évident que les outils nécessaires à répondre à nos interrogations scientifiques n'existaient pas encore et qu'il fallait les mettre au point par nous-mêmes. J'ai aussi entretenu de nombreuses collaborations avec des laboratoires de l'Université de Sherbrooke et d'ailleurs afin de toujours pousser plus loin les limites de nos connaissances et d'amener de nouveaux points de vue à nos problèmes techniques et scientifiques. Au cours de mes travaux de doctorat, nous avons constamment été à la limite de la technologie disponible afin de répondre à mes questions expérimentales. Le développement du séquençage de deuxième génération a grandement contribué à l'avancement de mon projet, mais demeure encore une limitation. J'ai espoir que les avancées des prochaines années permettront à mes successeurs de répondre à mes interrogations scientifiques tant qu'à l'importance de la possible diversité génétique que pourrait entrainer le remodelage de la chromatine des spermatides. Les nouvelles technologies de séquençage permettront sans aucun doute à la dDIP de devenir un outil essentiel dans l'étude de l'intégrité génomique dans de nombreux domaines, tels que la sénescence, le cancer, la toxicologie et l'évolution. De plus, ces nouvelles connaissances amèneront de

nouvelles avenues de traitements et certainement une meilleure compréhension de nombreux cas d'infertilité masculine encore inexpliqués.

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Annexes

Annexe 1 : The sperm nucleus : chromatin, RNA, and the nuclear matrix, Johnson *et al.* Reproduction (2011)

REVIEW

The sperm nucleus: chromatin, RNA, and the nuclear matrix

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Abstract

Within the sperm nucleus, the paternal genome remains functionally inert and protected following protamination. This is marked by a structural morphogenesis that is heralded by a striking reduction in nuclear volume. Despite these changes, both human and mouse spermatozoa maintain low levels of nucleosomes that appear non-randomly distributed throughout the genome. These regions may be necessary for organizing higher order genomic structure through interactions with the nuclear matrix. The promoters of this transcriptionally quiescent genome are differentially marked by modified histones that may poise downstream epigenetic effects. This notion is supported by increasing evidence that the embryo inherits these differing levels of chromatin organization. In concert with the suite of RNAs retained in the mature sperm, they may synergistically interact to direct early embryonic gene expression. Irrespective, these features reflect the transcriptional history of spermatogenic differentiation. As such, they may soon be utilized as clinical markers of male fertility. In this review, we explore and discuss how this may be orchestrated.

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Introduction

Unlike the vast size of the oocyte, the diminutive sperm may have initially seemed unlikely to carry information in excess of its genomic cargo. Indeed, our ability to appreciate the contrary only began to gradually develop over the last two decades. This has been due to several factors, primarily reflecting the distinct nuclear environment of the mature spermatozoon. The sperm genome is repackaged into a near crystalline state, which has proven resistant to dissection often likened to a 'tough nut to crack'. This extensive remodeling both protects the paternal genome and is requisite for the characteristic reduction in nuclear volume which occurs as the head takes on a unique shape (reviewed in Braun (2001) and Balhorn (2007)). The assumption that sperm occupy a limited developmental role compared to oocytes has in part been due to these physical constraints and the appropriate enabling physical, chemical, and biological technologies (Kierszenbaum & Tres 1975).

Despite the near complete packaging of the sperm genome as protamine (PRM)-associated DNA, it is increasingly clear that specific regions retain a somatic-like structure (reviewed in Miller *et al.* (2010)). In some cases, these regions are differentially marked by modified histones in a manner reminiscent of the epigenetic states observed in somatic or stem cells (Hammoud *et al.* 2009, Brykczynska *et al.* 2010). This feature of sperm chromatin has been suggested to influence the order that genes are repackaged into a nucleosomal bound state and/or expressed following fertilization (reviewed in Rousseaux *et al.* (2008)). Additionally, sites of histone retention are likely to provide insight into the transcriptional history of spermatogenesis.

RNAs produced during this prior window of transcription are retained in sperm and delivered to the oocyte. The biological role of these transcripts post-fertilization remains a subject of debate. Regardless of their function, several of these molecules are currently being developed as biomarkers of male fertility (Depa-Martynow *et al.* 2007, Jedrzejczak *et al.* 2007, Lalancette *et al.* 2009). Importantly, the notion of a sperm enriched in RNAs continues to expand with the isolation and characterization of a complement of male gamete small non-coding RNAs (sncRNAs; C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

A subset of sperm RNAs may also serve to structurally support the nuclear matrix. This proteinaceous network present in most cells functionally organizes the genome by binding discreet regions of DNA at sequences termed scaffold/matrix attachment regions (S/MARs). S/MAR binding partitions the genome into cell type-specific

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loop domains, which range in size from 30 to 110 kb in somatic cells (Vogelstein *et al.* 1980, Linnemann *et al.* 2009, Drennan *et al.* 2010) and from 20 to 50 kb in sperm (Ward *et al.* 1989, Barone *et al.* 1994, Nadel *et al.* 1995). Nucleosome-bound DNA maintained in mature sperm has been proposed to mark sites of nuclear matrix attachment in these cells. These structural markers likely correspond to the S/MARs anchoring the decondensed DNA loops of prior cell types and may serve to recapitulate paternal nuclear architecture in the zygote (Ward 2010).

The notion that the male gamete merely delivers paternal DNA to the oocyte is falling by the wayside. This reflects several developments pertaining to the interacting function of the three main structural genetic elements of the sperm nucleus: chromatin, RNA, and the nuclear matrix. In a manner accessible to all reproductive biologists, this review explores and discusses how this unique nuclear symphony may be conducted. As such, when appropriate, a role for paternal chromatin, RNA, and the nuclear matrix beyond the interior of the sperm nucleus is discussed in terms of potential impact on embryonic development. While not the primary focus of this review, one is also referred to several timely reviews discussing paternal imprinting, the transgenerational effects of germline mutations (Butler 2009, Nadeau 2009, de Boer *et al.* 2010) providing additional perspectives.

Sperm chromatin

Spermatogenesis is characterized by ordered histone replacement. As spermatogonia commit to this differentiative pathway, they have already begun to incorporate testis-specific histone variants into their chromatin (Meistrich *et al.* 1985, van Roijen *et al.* 1998). Synthesis and deposition of these proteins peak during meiosis (Kimmins & Sassone-Corsi 2005). Supported by the action of testis-specific histone variants, in round spermatids, the majority of histones are replaced first by the transition proteins (TNPs) and subsequently by PRMs. Some histone variants, as well as canonical histones, are maintained throughout the remaining stages of spermatogenesis (Shires *et al.* 1976, Seyedin & Kistler 1980, Gatewood *et al.* 1987, 1990, Witt *et al.* 1996, Chadwick & Willard 2001, Zalensky *et al.* 2002, Yan *et al.* 2003, Churikov *et al.* 2004*a*, reviewed in Churikov *et al.* (2004*b*), Tanaka *et al.* (2005) and Govin *et al.* (2007)).

Chromatin remodeling requires regulated post-translational modifications of histones including acetylation (Oliva & Mezquita 1982, Christensen *et al.* 1984, Grimes & Henderson 1984, Meistrich *et al.* 1992, Marcon & Boissonneault 2004), ubiquitination (Chen *et al.* 1998, Baarends *et al.* 1999, Lu *et al.* 2010), methylation (Godmann *et al.* 2007), and phosphorylation (Meyer-Ficca *et al.* 2005, Krishnamoorthy *et al.* 2006, Leduc *et al.* 2008*a*), and has been recently reviewed in the context of spermatogenesis (Rousseaux & Ferro 2009). Among these modifications, the best characterized to date is the global hyperacetylation of histones. Incorporation of these marks destabilizes nucleosomes in preparation for their replacement by the TNPs and ultimately by the PRMs (Pivot-Pajot *et al.* 2003, Kurtz *et al.* 2007).

Hyperacetylation is essential in mice and men as perturbation is correlated with defective spermatogenesis (Sonnack *et al.* 2002, Fenic *et al.* 2004). This is supported by the observation that species maintaining chromatin in a somatic-like state do not exhibit elevated levels of histone acetylation in sperm (Christensen *et al.* 1984). For example, trout spermiogenesis spans several weeks during which spermatids exhibit high steady state levels of hyperacetylation. Extended maintenance of this modification in the absence of protamination suggests that additional factors are needed to complete nuclear remodeling (Christensen *et al.* 1984, Csordas 1990). Even precocious hyperacetylation in *Drosophila* does not prematurely induce the histone to PRM spermatid transition (Awe & Renkawitz-Pohl 2010). There are several potential pathways regulating initiation of chromatin remodeling. However, inhibition of the ubiquitin–proteasome pathway by loss of an ubiquitin ligase can block global histone acetylation, degradation, and PRM deposition, resulting in sterility (Lawrence 1994, Roest *et al.* 1996, Lu *et al.* 2010). In these studies, mature spermatozoa were low in number and exhibited altered morphologies, reminiscent of teratozoospermia. Indeed, microarray analysis of sperm RNAs from teratozoospermic patients presents a severe disruption of the ubiquitination pathway (Platts *et al.* 2007).

During murine and human protamination, histones are replaced first by the TNPs then subsequently displaced by the PRMs (Balhorn *et al.* 1984). Binding of these small arginine-rich proteins to the negatively charged phosphodiester backbone of the double helix abolishes the electrostatic repulsion between the proximal chromatin strands resulting in the formation of a toroid loop (Hud *et al.* 1993). Containing ~50 kb of DNA, these donut-shaped structures are further stabilized by inter- and intramolecular disulfide bridges compressing the genome into a semi-crystalline state as the spermatozoon transits through the epididymis (Golan *et al.* 1996). The resulting mature human sperm nucleus is now condensed to 1/13th the size of that of the oocyte (Martins & Krawetz 2007*b*).

Despite compaction, the restructured paternal chromatin retains a hierarchical layer of genomic organization (Zalensky & Zalenskaya 2007). Reminiscent of somatic cells, individual chromosomes are not randomly positioned, but occupy rather distinct territories preferentially localized within the nucleus with respect to one another (Hazzouri *et al.* 2000, Zalenskaya & Zalensky 2004). The positioning of chromosome territories in porcine spermatozoa is first observed in spermatids. Preceding meiosis, their relative position resembles that seen in somatic cells (Foster *et al.* 2005). It has

been proposed that within sperm, each chromosome territory generally adopts a 'looped hairpin' conformation orienting its centromere towards the nuclear interior and distal telomeres towards the periphery (Mudrak *et al.* 2005).

Nuclear remodeling has been proposed to serve three functions (Braun 2001). First, the reduced size and shape of the sperm nucleus yields a hydrodynamic structure that is predictive of fertility in bulls and red deer (Ostermeier *et al.* 2001, Malo *et al.* 2006, Gomendio *et al.* 2007). Second, protamination renders the majority of the sperm genome resistant to nuclease attack, irradiation, and shearing forces (Kuretake *et al.* 1996, Wykes & Krawetz 2003, Rathke *et al.* 2010). Presumably, both features were evolutionarily optimized to protect the paternal genome while traversing the female reproductive tract en route to the oocyte. Third, although a subject of debate, the selective post-meiotic retention of histones provides the zygote a dichotomous chromatin package that could serve to preferentially poise regions for early use (Gatewood *et al.* 1987, Hammoud *et al.* 2009, Brykczynska *et al.* 2010).

Murine spermatozoa organize about 1–2% of their genome with nucleosomes (Balhorn *et al.* 1977, Brykczynska *et al.* 2010), whereas up to 15% of human sperm DNA is packaged in this manner (Tanphaichitr *et al.* 1978, Gusse *et al.* 1986, Gatewood *et al.* 1990). Interrogation of isolated nucleosome-associated sequences demonstrated that some of these genomic regions included imprinted regions (Banerjee & Smallwood 1998), telomeres (Pittoggi *et al.* 1999, Zalenskaya *et al.* 2000), retroposon DNA (Pittoggi *et al.* 1999), and specific gene loci (Gardiner-Garden *et al.* 1998, Pittoggi *et al.* 1999, Wykes & Krawetz 2003). Lacking comparable nucleosomal enrichment, the centromeric and pericentromeric regions of mammalian sperm present a mix of nucleosomes and PRMs (Wykes & Krawetz 2003). Specifically, these regions retain modified histones such as H3K9me3 as well as the histone variants CENPA and H2A.Z (Palmer *et al.* 1990, Zalensky *et al.* 1993, Hammoud *et al.* 2009). Together these observations led to the hypothesis that the maintenance of nucleosomes at specific sites may prime discreet regions for use shortly after fertilization. Initial support for this premise came from the finding that, in human sperm, histones bind DNA in a sequence-specific manner around gene regulatory regions (Gatewood *et al.* 1987, Wykes & Krawetz 2003).

Studies reporting the *in situ* localization of nucleosome-associated genomic regions in the sperm should be met with caution. The compact nuclear environment of the spermatozoa cannot be accurately interrogated by immunofluorescence without prior membrane destabilization and chromatin decondensation. Treatment may skew interpretations as decondensation alters the position of nuclear elements (van Roijen *et al.* 1998). With this caveat, in human spermatozoa, core histones as well as testes-specific histone variants have been observed within the basal portion of the nucleus proximal to the tail (Zalensky *et al.* 2002, Li *et al.* 2008). In contrast, histone H2B as well as nucleosome-associated telomeric regions exhibits a partially overlapping punctuate pattern throughout the nucleus (Gineitis *et al.* 2000, Zalensky *et al.* 2002). In the mouse, telomeres are bound by linker H1, which is absent in human sperm, and appear localized to the periphery (Gatewood *et al.* 1990, Pittoggi *et al.* 1999). It cannot be excluded that these results primarily reflect nuclear access. As an additional point of comparison, the canonical histones found in spermatozoa of the evolutionarily distant marsupial, *Sminthopsis crassicaudata*, are also peripherally located (Soon *et al.* 1997). Regardless of the limitations inherent to these studies, it is generally agreed that the nucleoprotamine and nucleohistone components in sperm are discreetly partitioned (van der Heijden *et al.* 2006, Li *et al.* 2008).

Recent advances in methods of genome-wide analysis now allow for the detection of histone-enriched regions at the primary sequence level. Using CGH tiling arrays, it was established that histone-bound DNA is associated with gene-dense regions and enriched for developmentally regulated promoters as well as CTCF binding sites (Arpanahi *et al.* 2009). In parallel, next generation sequencing (NGS) provided a significantly higher resolution analysis (Hammoud *et al.* 2009). Nucleosome-associated sequences exhibited a modest enrichment within the promoters of developmentally important genes including embryonic transcription factors and signaling pathway components, as well as microRNA (miRNA) and imprinted gene clusters. Independent analysis has demonstrated that internal exons also display significantly greater histone enrichment than flanking intronic sequences (Nahkuri *et al.* 2009). Outside of promoters, histones were found to be distributed, at low levels, throughout the genome. This pattern of nucleosome retention has recently been confirmed using similar NGS technologies (Brykczynska *et al.* 2010).

Combining chromatin immunoprecipitation (ChIP) and NGS (i.e. ChIP-seq) revealed that developmentally regulated promoters may be bivalently marked by H3K4me2/3 and H3K27me3 (Hammoud *et al.* 2009, Brykczynska *et al.* 2010). The bivalent promoter is a hallmark of developmentally regulated stem cell genes and has recently been observed in zebrafish blastomeres (Vastenhouw *et al.* 2010). In addition to harboring sites of both active and repressive histone modifications, bivalent promoters are often bound by RNA polymerase and are therefore poised for expression. To date, this correlation has not been established in mature sperm. The coordinated removal of repressive H3K27me3 throughout differentiation permits the initiation of transcription, providing temporal and spatial

control of gene expression. Bivalent promoters might reflect the male contribution to early gene expression (Petronis 2010).

Alternatively, differential enrichment of histone modifications within specific ontological categories of promoters, and not bivalency, may regulate early embryonic gene expression (Brykczynska *et al.* 2010). In human sperm, H3K4me2 marked promoters of genes associated with spermatogenic and housekeeping processes, whereas H3K27me3 was enriched within the promoters of developmentally regulated genes expressed following implantation or in differentiated cells. Furthermore, the degree to which a promoter was occupied by H3K27me3 positively correlated with repression of the corresponding gene during early mouse embryonic development. Together these results argue that the retention of the repressive H3K27me3 modification at specific promoters in human sperm may provide a paternal and possibly transgenerational mark (Petronis 2010).

The two modes of paternally derived epigenetic promoter regulation introduced above, bivalency and differential enrichment of modified histones, are likely present in sperm of both mice and men. As illustrated in Fig. 1, the use of one mechanism in lieu of the other would be expected to hinge on shared spermatogenic transcriptional requirements and the species-specific timing of zygotic genome activation (ZGA).

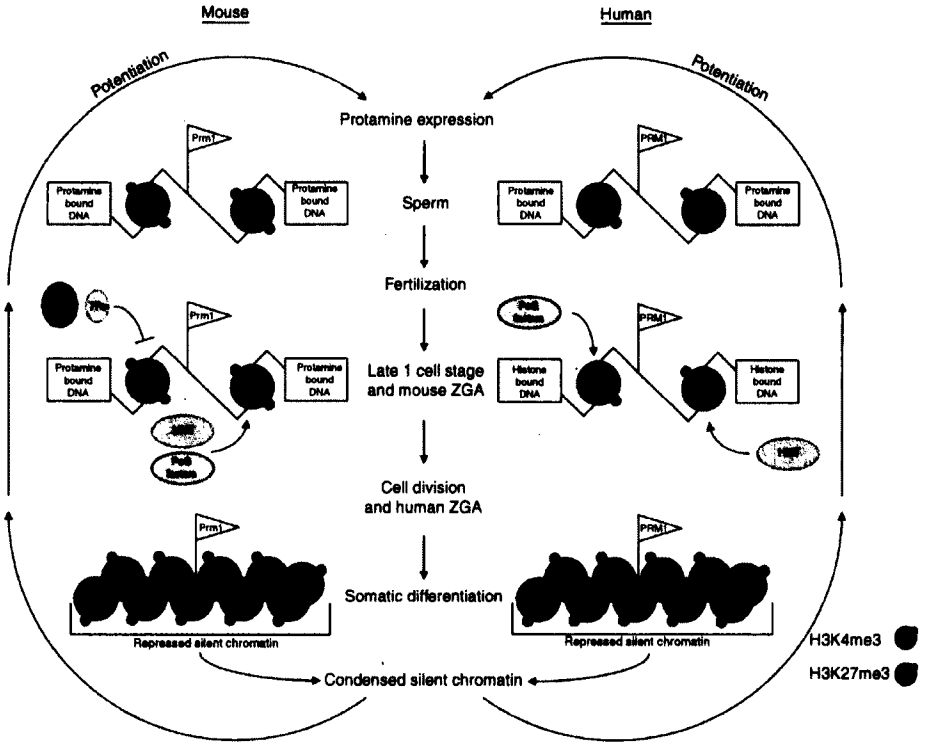

Figure 1 The potential influence of zygotic genome activation on paternal chromatin structure. In mouse and human sperm, the protamine genes are bound by nucleosomes residing within a potentiated DNase I-sensitive domain. These regions are differentially marked by modified histones in each species. In mouse, the bivalently marked spermatogenic promoters may reflect the early initiation of zygotic expression at the late one-cell stage. Recruitment of transcriptional machinery (RNA polymerase, RNA Pol II; transcription factors, TFs) is coincident with the activation of silencing pathways (histone methyltransferases, HMTs; polycomb factors, PcG). The retention of the silencing H3K27me3 mark in promoters may prevent detrimental expression prior to gene silencing. In comparison, human zygotic genome activation occurs at the four- or eight-cell stage. This affords the embryo time to silence these genes, which in sperm are marked with the active H3K4me3 modification lacking the repressive mark. In both species, the protamine domain remains silenced throughout differentiation by adopting a highly condensed chromatin conformation. During male gametogenesis, this region becomes potentiated in spermatocytes prior to its expression in round spermatids.

Whereas promoters of potent developmental regulators in sperm from both species are primarily associated with repressive histone modifications, spermatogenic genes are bivalently marked in murine but not in human sperm (Brykczynska *et al.* 2010). The former reflects a shared need for early repression of developmental gene expression. The presence of active modifications in mouse and human spermatogenic promoters likely corresponds to the transcriptional history of these silent cells. In mouse, these regions are marked by repressive histone modifications to ensure their appropriate regulation following fertilization. Mice initiate ZGA late in the one-cell embryo (Schultz 2002, Minami *et al.* 2007), concurrent with DNA replication (Aoki *et al.* 1997). This is paralleled by an increase in the levels of H3K27me3 within the paternal pronuclei through the activity of polycomb group (PcG) proteins (Santos *et al.* 2005). Prior to this, H3K27me3 cannot be microscopically detected in paternal chromatin of the one-cell fertilized oocyte (Santos *et al.* 2005, van der Heijden *et al.* 2005, Puschendorf *et al.* 2008). Methylated sperm histones are expected to remain reflecting the lack of histone demethylase activity in either the oocyte or the zygote (Puschendorf *et al.* 2008). This is likely essential to ensure proper transcriptional regulation from the paternal chromatin during this initial wave of ZGA. Concomitantly, the male pronucleus exhibits a higher level of transcriptional activity (Aoki *et al.* 1997), an increased concentration of transcription factors (Worrad *et al.* 1994), and a more transcriptionally permissive chromatin structure compared to the female pronucleus (Adenot *et al.* 1997, Schultz 2002). It is reasonable to assume that the presence of sperm-derived H3K27me3 within the bivalent promoters of the paternal spermatogenic genes enables the propagation of the polycomb repressive mark preventing their transcription (Margueron *et al.* 2009, Brykczynska *et al.* 2010). This would be expected to block transcription factor recruitment and subsequent expression. Repression of these genes is necessary as expression of PRM1, which is bivalently marked in mouse sperm, would likely perturb further development (Lee *et al.* 1995). Indeed, mutant mice lacking the methyltransferase activity (required to propagate H3K27me3) do not progress past early development (O'Carroll *et al.* 2001). Though undoubtedly this mutation is responsible for a wide range of developmental defects (Erhardt *et al.* 2003, Puschendorf *et al.* 2008), it would be informative to probe these late zygotic mutants for expression of those spermatogenic genes marked by a bivalent promoter in wild-type sperm. Comparatively, the delayed ZGA of humans (Braude *et al.* 1988) should permit PcG-mediated repression of orthologous spermatogenic promoters altering the paternally derived poised chromatin structure. The inability to detect trimethylated paternal H3K27 in G2 tripronuclear zygotes suggests that deposition of this modification occurs sometime after the first cleavage event but before the start of embryonic gene expression at the four- to eight-cell stage (van der Heijden *et al.* 2009).

The number of histone variants and associated secondary modifications found in mammalian sperm has greatly increased in the last two decades (reviewed in Rousseaux & Ferro (2009) and Carrell & Hammoud (2010)). Detection of these proteins following fertilization has proven challenging for several reasons. First, the amount of histone-associated chromatin in sperm is limited, ranging from 1 to 15% in mice and men respectively. Secondly, epitopes may be inaccessible prior to decondensation limiting detection. Thirdly, deposition of maternal histones, which are virtually indistinguishable from their paternally derived counterparts, directly coincides with sperm chromatin decondensation (van der Heijden *et al.* 2005, 2008). This is best exemplified by the replication-independent histone variant H3.3. Though, present in mature sperm (Gatewood *et al.* 1990), H3.3 is not microscopically detectable in paternal chromatin until maternally derived histones are deposited at the start of decondensation (van der Heijden *et al.* 2005, Torres-Padilla *et al.* 2006), the prevalence of this variant in paternal chromatin is conserved and likely essential to remodeling as mutation of the HIRA chaperone blocks H3.3 incorporation precluding decondensation in *Drosophila* zygotes (Loppin *et al.* 2005, Ooi & Henikoff 2007).

Despite the difficulty in detecting nucleosome-bound DNA delivered by sperm, some paternally derived modified histones and histone variants have been observed following fertilization. These include both H4K8ac and H4K12ac (van der Heijden *et al.* 2006) as well as the testis-specific variants H2AL1 and H2AL2. First detected in the centromeres of spermatids, these variants remain enriched in heterochromatin until displaced from paternal DNA shortly after fertilization (Wu *et al.* 2008). In contrast to histone, H3 replication-dependent variants H3.1 and H3.2 (Tagami *et al.* 2004) are detected following fertilization in decondensed sperm chromatin prior to DNA synthesis, though in much lower abundance than in maternal chromatin (van der Heijden *et al.* 2005, 2008). These sperm-derived proteins are detected until the zygotic S phase initiates, at which point they become indistinguishable from their newly incorporated maternal counterparts (van der Heijden *et al.* 2008).

As described above, many sites of histone enrichment likely have no impact on the zygote and simply reflect the transcriptional history of these silent cells. Indeed, this has been hypothesized to be the role of H3K4me2 in human sperm (Brykczynska *et al.* 2010). A comparison of the genic regions, which remain associated with nucleosomes following spermiogenesis to those RNAs retained in sperm, may help identify this population of promoters.

RNA in sperm

It is now accepted that mature spermatozoa harbor a distinct population of RNAs. The biological role of these transcripts largely remains unknown. Undoubtedly, some of the transcripts retained in sperm represent products expressed in various spermatogenic cells. The proposed functions of others include the regulation of early embryonic gene expression and stabilization of the nuclear matrix.

Owing to the observation that mature mammalian sperm are transcriptionally quiescent (Kierszenbaum & Tres 1975), the presence of mRNAs in these cells was originally thought to represent incomplete expulsion of cytoplasmic elements during nuclear condensation. Indeed, sperm do contain remnants of their developmental expression profile, which seemingly serve no purpose in the mature gamete. Furthermore, some of these RNAs are highly abundant in sperm and expected to be detrimental to the embryo (Lee *et al.* 1995). In this regard, the PRM transcripts are the most conspicuous. Following their transcription in round spermatids, these RNAs are translationally repressed and stored as inactive messenger ribonucleoprotein particles prior to remodeling (Kleene 1989, Kwon & Hecht 1993). Loss of this repression causes premature PRM translation in these cells. The subsequent developmental arrest is likely due to precocious PRM-dependent nuclear condensation. Nuclei from these cells, such as those from mature spermatozoa, are sonication resistant (Lee *et al.* 1995, Kuretake *et al.* 1996). The affinity of PRMs for DNA coupled with the enduring abundance of these transcriptionally repressed RNAs in sperm presents a potentially precarious situation to the zygote. However, failure to detect these transcripts soon after fertilization by ICSI or round spermatid injection despite the persistence of other sperm RNAs (Ziyyat & Lefevre 2001, Avendano *et al.* 2009) suggests that the zygote has evolved mechanisms and pathways to cope with this consequence of paternal genome compaction.

An evolutionarily distant precedent for such a mechanism has recently been observed in *Arabidopsis* (Bayer *et al.* 2009). Expressed during male gametogenesis, short suspensor (*SSP*) transcripts are translationally repressed and stored in pollen. Following fertilization, repression is relieved, and the *SSP* gene product undergoes zygotic translation. Sufficient accumulation of this protein in the seed activates a MAP kinase signaling cascade prompting the first cell division. In this model, embryo patterning is temporally linked to fertilization by a paternally contributed mRNA. Whether such regulation exists in other species is the subject of intense debate. It should be noted that parthenogenetic mice survive to adulthood and produce offspring in the absence of a paternal factor (Kono *et al.* 2004, Kawahara *et al.* 2007). However, efficient generation of these embryos requires the deletions of both copies of two paternally methylated imprinting control regions. Furthermore, the possibility that transgenerational affects may present must be considered.

Regardless of species, if paternally derived mRNAs are to impact embryogenesis, they must, like *SSP*, first be selectively stored in sperm. Aiding in the detection of transcripts that fulfill this prerequisite has been the development of high throughput technologies. Accordingly, the use of microarrays to screen RNA profiles from human sperm and preceding cell types provided the first evidence for the existence of a sperm-specific transcript (Ostermeier *et al.* 2002). Interestingly, in the bull, despite a high percentage (~37%) of transcripts being shared between cell types, the majority of mRNAs (59%) present in round spermatids are absent in the mature gamete (Gilbert *et al.* 2007). In addition to the selective loss of transcripts, ~120 RNAs were enriched in sperm compared to spermatids.

Comparing transcripts retained in sperm from pooled and individual human ejaculates suggested the existence of a common spermatozoal mRNA fingerprint (Ostermeier *et al.* 2002). Intriguingly, the RNA profile shared among these fertile donors included transcripts implicated in fertilization and development (Ostermeier *et al.* 2002). Some of these mRNAs are absent in human and hamster oocytes but are present in embryos (Kocabas *et al.* 2006, Avendano *et al.* 2009). Several laboratories have since independently observed these RNAs in zygotes following heterologous fertilization (Ostermeier *et al.* 2004, Avendano *et al.* 2009). These findings suggest that in a species-specific manner, some mRNAs are selectively retained in mature spermatozoa, delivered to the oocyte, and persist in the zygote.

Early investigations comparing sperm RNAs from pooled and individual fertile donors identified few, if any, differences between samples (Ostermeier *et al.* 2002). However, recent technological advances have resolved their variability (Lalancette *et al.* 2009). This may be due to the inherent heterogeneity of sperm (Lefievre *et al.* 2007, Lewis 2007), as evidenced by the normalization of transcript profiles following sperm selection (Garcia-Herrero *et al.* 2010). For example, when sperm mRNA profiles from 24 fertile individuals (Lalancette *et al.* 2009) were clustered using standard microarray comparative techniques, groups of samples clustered to differing degrees. However, a total of 453 transcripts were detected above background in all 24 samples. Of these, 30 'transcript pairs' were identified on the basis that although the signal intensity of the transcripts changed from one sample to another, this change occurs in parallel, such that the signal ratio of two transcripts in a pair was relatively stable across all 24 samples. This method of microarray analysis has since been utilized to evaluate tumor gene networks for diagnosis and prognosis, which also exhibit considerable variability between individual transcript profiles (Platts *et al.* 2010). Interestingly, transcripts known to be translationally repressed in mature spermatozoa were

detected, though none formed 'stable pairs'. Whether the paired transcripts are also translationally repressed and by what mechanism(s) remains to be elucidated. Irrespectively, the non-random enrichment of RNAs in sperm suggests that these RNAs are not solely remnants of transcription. Though some paternal transcripts may function in the early embryo, it seems unlikely that all of the selectively retained mRNAs stored by the male gamete should impact development. What other functions can be ascribed to these transcripts?

With the exception of PLC zeta (Parrington *et al.* 1999), it is not known whether the proteins corresponding to the majority of these retained transcripts are also present in mature spermatozoa and what proteins survive delivery to the oocyte. Comparing these mRNAs to the still developing sperm proteome (Baker *et al.* 2008, Oliva *et al.* 2008, Baker & Aitken 2009, Nixon *et al.* 2009) would help guide future investigations concerning the functional significance of the sperm-retained transcripts. This approach was recently used to demonstrate the selective retention of mRNAs expressed from the non-recombining region of the human Y chromosome (Yao *et al.* 2010).

Analysis of the sperm transcripts cannot be confined solely to mRNA. Acceptance of RNA in sperm was well timed with the discovery of RNAi (RNA interference) and the subsequent appreciation for the biological role of sncRNAs and their initial identification in spermatozoa (Moldenhauer *et al.* 2003). sncRNAs are approximately between 18 and 30 nucleotides in size, and classified in families according to their biogenesis (Moazed 2009). In somatic cells, these transcripts contribute to gene regulation and chromatin structure, and inhibit transposition. Two of the most studied classes of sncRNAs are the small interfering RNA (siRNA) and the miRNA families. These molecules of 20–24 nucleotides are processed from hairpins through pathways involving DICER, an endoribonuclease of the RNase III family. Data pertaining to these male germline transcripts in testis have recently been reviewed (Papaioannou & Nef 2010). However, they remain largely uncharacterized in mature sperm (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

In addition to siRNAs and miRNAs, the testis expresses piwi-interacting RNAs (piRNAs). These transcripts of 26–30 nucleotides are produced in a DICER-independent manner that does not require double-stranded RNA folding (reviewed in Klattenhoff & Theurkauf (2008) and Ghildiyal & Zamore (2009)). Complementary to transposons, these RNAs repress the rate of transposition, thereby protecting the genome from mobile elements. Currently, the presence of these small RNAs has been demonstrated in spermatogenic cells (reviewed in Lau (2010)) where their function is essential to spermatogenesis (Deng & Lin 2002, Kuramochi-Miyagawa *et al.* 2004). Though assumed to be absent from the mature gamete, a restricted set of piRNAs may be retained in human spermatozoa (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

The demonstration that miRNAs, and other small RNAs, are retained in the mammalian sperm nucleus and similar to mRNAs delivered to the zygote has ignited much debate (Ostermeier *et al.* 2005, Amanai *et al.* 2006, Yan *et al.* 2008, Curry *et al.* 2009). The absence of transcriptional activity in sperm has prompted the hypothesis that paternally contributed miRNAs may regulate early embryonic expression influencing offspring phenotype (Rassoulzadegan *et al.* 2006, Grandjean *et al.* 2009). However, the current pace at which novel sncRNAs can be identified by high throughput sequencing technologies far surpasses the ability to determine their biological role, if any. A detailed catalog and analysis of the sperm RNA are wanting.

Towards this end, a recent study has provided the first glimpse of the complexity of this component of the sperm transcriptome (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations). Small sperm RNAs (<200 bp) purified from single ejaculates from three fertile donors were subjected to high throughput sequencing. Isolated sncRNAs comprised ~3 of the 10–20 fg of the RNA found in an individual sperm (Krawetz 2005). The average length of these transcripts was 18 bp. Sequenced reads were classified as either aligning uniquely or to multiple locations (two to ten sites) throughout the genome. Greater than half of the RNAs (58%) mapped to multiple locations in the genome. The majority (70%) of uniquely mapped reads correspond to novel sncRNAs primarily derived from intronic and intergenic regions. The miRNAs were a small percentage (3%) of the known sncRNAs in those that uniquely aligned to the genome as well as those that aligned to multiple locations.

Though miRNAs were the first class of sncRNAs observed in mammalian sperm, they account for relatively few of the sncRNAs shared between donors. However, there may only be limited opportunities for post-transcriptional regulation of early development by miRNAs. Indeed, recent reports have established that this pathway is strongly down-regulated during oocyte maturation and not required for preimplantation development (Ma *et al.* 2010, Suh *et al.* 2010). Perhaps, paternal miRNAs and other short RNA species delivered to the zygote bypass their canonical regulatory pathway altogether. In somatic cells, sncRNAs and short RNAs (~50–200 nt) bind to complimentary promoter regions silencing gene transcription through the recruitment of PcG proteins and repressive histone marks (Kim *et al.* 2008, Kanhere *et al.* 2010). The majority of miRNAs identified in sperm (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations) originate from promoter regions. These transcripts may bind to paternal DNA during nuclear remodeling such that they are delivered to the oocyte in association with their targeted *cis* sequences presumably influencing their local chromatin structure.

The sperm nuclear matrix

As discussed above, appreciation that the mature spermatozoon is more than a vehicle for the delivery of inert DNA has evolved with the acceptance that distinct regions of the paternal genome remain nucleosome-bound (Gardiner-Garden *et al.* 1998, Wykes & Krawetz 2003, Arpanahi *et al.* 2009, Hammoud *et al.* 2009). Complementing this development was the discovery that sperm also deliver a suite of RNAs to the oocyte (Ostermeier *et al.* 2004). Both have contributed to expanding the post-fertilization genetic influence of the male gamete. Our understanding of how these elements coalesce to potentially influence embryonic development would not be complete without considering the RNA containing nuclear matrix (Malyavantham *et al.* 2008).

In most cells, DNA is functionally organized by a proteinaceous network termed the nuclear matrix (Cook & Brazell 1975, Ward *et al.* 1989, Choudhary *et al.* 1995, Kramer & Krawetz 1996, Heng *et al.* 2004, Linnemann & Krawetz 2009*a*, 2009*b*, Ward 2010). When isolated and viewed by electron microscopy, this ultrastructure resembles the fibrous architecture of the cytoskeleton (Comings & Okada 1976, Berezney & Coffey 1977, Fey *et al.* 1984). The list of proteins comprising the nuclear matrix is vast and to some degree cell type-dependent (reviewed in Mika & Rost (2005) and Albrethsen *et al.* (2009)). Associated with the sperm nuclear matrix are various structural proteins such as actin, myosin, and lamin B, as well as transcription factors and chromatin modifiers such as the topoisomerases (Moss *et al.* 1993, Carrey *et al.* 2002, Ocampo *et al.* 2005, Har-Vardi *et al.* 2007). Only recently, spermatozoa have, similar to somatic cells, been shown to contain a population of RNAs that associate with the nuclear matrix (reviewed in Lalancette *et al.* (2008)). Perhaps these transcripts fulfill a structural role.

The ordered positioning of chromatin within the nucleus results from attachment of discrete S/MAR sequences to this network of proteins and RNAs. Chromatin anchored to the matrix by S/MARs forms cell type-specific loop domains within interphase nuclei. Differential matrix attachment has been shown to coincide with DNA synthesis (Adom & Richard-Foy 1991, Anachkova *et al.* 2005, Courbet *et al.* 2008) and contribute to cell type-specific gene expression (Heng *et al.* 2004, Linnemann & Krawetz 2009*a*, 2009*b*). Despite the absence of replication and transcription in sperm, evidence suggests that the nuclear matrix both structurally orders and imparts function to the paternal genome.

Studies investigating the role of the sperm nuclear matrix commonly require chromatin to be relieved of PRM compaction. Treating sperm with alkali or high concentrations of buffered salts in the presence of a reducing agent such as dithiothreitol (DTT) displaces PRMs and the remaining histones. However, the strong interactions between DNA and nuclear matrix appear preserved (Ward *et al.* 1989). Once decondensed, the otherwise unconstrained DNA loops radiate out from the matrix forming a diffuse weakly staining halo around a brightly staining central region. The strong fluorescent signal corresponds to chromatin at the bases of the DNA loop domains which remain associated with the nuclear matrix (Kramer & Krawetz 1996). Similar extraction protocols are commonly used with somatic cells; though due to the absence of disulfide bonds, reducing agents are not required (Berezney & Coffey 1977, Linnemann *et al.* 2009, Drennan *et al.* 2010).

Studies of sperm nuclear halos have yielded estimates of the length of individual DNA loops (20–50 kb), which approximately correspond to the amount of DNA within an individual toroid (Ward *et al.* 1989, Hud *et al.* 1993, Barone *et al.* 1994, Nadel *et al.* 1995). This observation has prompted the notion that these discrete subunits of DNA are directly related (Ward 1993). It was proposed that during spermiogenesis, individual DNA loop domains condense to form single toroid structures (Ward 2010). Each toroid is then tethered to the nuclear matrix by adjacent nuclease-sensitive linker regions. These regions are expected to correspond to the S/MARs flanking DNA loop domains. Nuclease sensitivity would be ensured if these sequences escaped protamination. Accordingly, following sperm chromatin decondensation, these linker regions may be used to recapitulate the paternal DNA structure (Ward 2010).

Support for this model comes from the observation that spermatozoa possess endogenous nuclease activity that releases 50 kb DNA fragments (Sotolongo *et al.* 2005). Unlike the proposed nuclease-sensitive linker regions, the PRM-bound sequences would be shielded from degradation. Preferential digestion of the chromatin tethers would release the toroids, each of which contains a DNA sequence of approximately uniform length.

In addition to partitioning the sperm genome, the nuclear matrix may serve as a platform for the transgenerational inheritance of paternal chromatin structure. The proposal that matrix-associated linker regions in sperm may be recycled as embryonic S/MARs (Ward 2010) demarcating the initial embryonic replicons is broadly evidenced by the chromatin architecture of embryonic stem cells (ES cells). Unconstrained DNA loops in mammalian sperm and ES cells are reduced in size compared to those present in the liver or brain (Klaus *et al.* 2001, Ward 2010). The large widely spaced chromatin loops of differentiated mammalian cells are also observed in *Xenopus* erythrocytes. Nuclei from these cells incubated with M-phase egg extract remodel their chromatin structure to resemble the condensed narrowly space DNA loops of sperm and early embryonic cells. Once remodeled, these nuclei replicate their DNA at an efficiency and rate similar to that of the undifferentiated cells (Lemaitre *et al.* 2005). This activity is dependent on TOP2 as well as acetylated H3/4 (Adenot *et al.* 1997, Shaman *et al.* 2006). These results

suggest that the ordered positioning of chromatin domains by the sperm nuclear matrix persists in the early embryo and directs initial DNA synthesis.

Additional evidence for the inheritance of sperm DNA architecture has been garnered. Experimental disruption of the sperm nuclear matrix by treatment with detergent precludes embryogenesis following ICSI (Ward *et al.* 1999). Injection of intact sperm nuclear halos into oocytes supports the formation of male pronuclei capable of DNA replication. Similar results are achieved after restricted endonuclease digestion of extracted loop domains prior to ICSI. Maintenance of MAR sequences in conjunction with an intact nuclear matrix was sufficient to support the formation of the male pronucleus and subsequent paternal DNA replication. However, neither occurred when oocytes were injected with isolated DNA, DNase I-digested nuclear matrices, or both in parallel (Shaman *et al.* 2007). The necessity of the interaction between MARs and the nuclear matrix was confirmed by inducing TOP2-mediated cleavage presumably at toroid linker regions prior to ICSI. Loss of this association resulted in irreversible degradation of paternal DNA by as yet unidentified factors (Shaman *et al.* 2006). Several reports suggest a role for TOP2 after fertilization during sperm decondensation and pronuclear formation. However, it is not clear whether this activity in the oocyte is due to paternally or maternally derived enzyme (Bizzaro *et al.* 2000, St Pierre *et al.* 2002, Tateno & Kamiguchi 2004). Regardless, inheritance of an intact sperm nuclear matrix, regulated by TOP2, is expected to be essential to the initial stages of development as it likely orders the paternal chromatin structure.

Support for the hypothesis that the sperm nuclear matrix mediates a form of non-genetic information between parent and offspring has also been inferred from the studies of transgenerational genetic instability following germline exposure to toxins or radiation (reviewed in de Boer *et al.* (2010)). Chronic paternal exposure to low doses of cyclophosphamide (CPA) is correlated with an altered sperm nuclear matrix protein profile as well as abnormal chromatin condensation (Codrington *et al.* 2007*a*, 2007*b*). Pairing treated sires with healthy mares increased preimplantation loss as well as developmental defects. These were correlated with precocious DNA decondensation, an increase in DNA damage, perturbed gene expression, and changes in the timing of ZGA (Harrouk *et al.* 2000*a*, 2000*b*, 2000*c*, Grenier *et al.* 2010). These effects cannot be reconciled by the altered composition of the sperm nuclear matrices alone. Chronic exposure of post-meiotic spermiogenic cells to CPA results in varying types of DNA damage (Codrington *et al.* 2004). The lack of DNA repair in post-meiotic cells propagates these errors. The effects of CPA might be exacerbated by changes to higher order chromatin structure including reordered associations between S/MARs and the nuclear matrix, as these interactions are thought to be essential to early development.

Additional evidence for the sperm nuclear matrix influencing male fertility has been provided (Barone *et al.* 2000, Ankem *et al.* 2002). Infertile cryptorchidic patients presented with sperm nuclear matrix instability. Though hampered by a small sample size, this study supports the view that evaluation of sperm nuclear matrix stability could be informative in certain cases of male factor infertility. Similarly, the level of sperm DNA fragmentation may discriminate between damage to chromatin associated with the nuclear matrix, the proposed toroid linker regions, and that of the toroid DNA itself (Ward 2010). The role of DNA damage and its use in predicting male fertility have been reviewed elsewhere (Leduc *et al.* 2008*b*, Lewis *et al.* 2008, Aitken & Koppers 2011, Barratt *et al.* 2010).

Demonstrating transgenerational inheritance of paternal chromatin structure requires delineation of those DNA sequences associated with the nuclear matrix in sperm and the paternal pronucleus. Though a direct comparison is limited to model species, investigation of these interactions in human sperm is underway. Instrumental to this effort has been the increased sequence resolution afforded by newer high throughput technologies. These include the development of unique genomic array system capable of simultaneously and specifically assaying the single copy transgenic human PRM domain in addition to the endogenous locus (Johnson *et al.* 2011). Utilizing these methods, similar studies have been reported in varied somatic cell types (Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al.* 2007, 2009, Drennan *et al.* 2010). Preliminary analysis of the human sperm nuclear matrix from four donors has yielded intriguing results (Fig. 2A and B). Following extraction with 2 M NaCl and 10 mM DTT, in the presence of 10 mM EDTA, unconstrained DNA loops were released from isolated sperm nuclear matrices by EcoRI digestion. Matrix- and loop-associated DNA fractions were separated by centrifugation, labeled, and competitively hybridized to genomic tiling arrays. Analysis was confined to the PRM locus (Fig. 2). In agreement with previous studies, the coding regions of the domain reside within a nuclease-sensitive loop, which is anchored to the nuclear matrix by flanking S/MARs (Choudhary *et al.* 1995, Kramer & Krawetz 1996). This conformation reflects the prior expressive status of the locus which first becomes potentiated in pachytene spermatocytes (Kramer *et al.* 2000). Interestingly, the S/MARs display a degree of variance between the donors (Fig. 2B) and are comparatively distal of those previously observed (Choudhary *et al.* 1995, Kramer & Krawetz 1996). The majority of these regions show negligible sperm histone enrichment in contrast to the promoters and exons of the PRM locus. However, the large sequence block identified as the 3′ MAR in this study does appear to be strongly bound by nucleosomes,

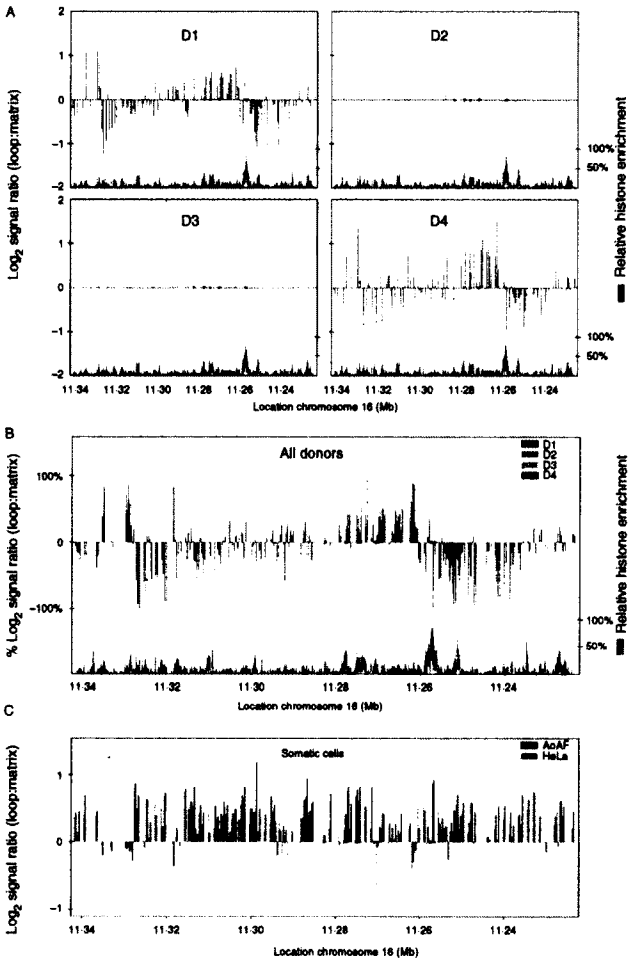

Figure 2 Nuclear matrix association within the protamine locus of sperm and somatic cells. Genomic regions in sperm associated with DNA loops or the nuclear matrix within a ~120 kb region of human chromosome 16 (chr 16: 11 223 803–11 341 499) are displayed as Log_2 values (loop/matrix). This region contains the complete protamine domain as well as the neighboring *SOCS1* gene. Genes are denoted by black arrows: *PRM1>PRM2>PRM3>TNP2>SOCS*. The relative histone enrichment across this region is illustrated in blue (GEO Series GSE15690, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15690). (A) Nuclear matrices were extracted from sperm from four fertile donors. Following EcoRI digestion, matrix- and loop-associated DNA fractions were labeled and competitively hybridized to Nimblegen CGAR0150-WHG8 CGH arrays. Loop- or matrix-association was determined as previously described (Linnemann *et al.* 2007). (B) Composite of percent normalized values from all four fertile donors. (C) Loop- and matrix-associated DNA fractions from HeLa and AoAF cells were identified as previously described (Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al.* 2009).

though this is likely due to the presence of the SOCS1 promoter. This entire region shares a high degree of synteny with sequence downstream of the mouse PRM domain which functions as a MAR in spermatids (Martins & Krawetz 2007*a*). This region also contains a 3′ boundary element that is essential for full expression of the human PRM genes (Martins *et al.* 2004). Mutations in this region have been correlated with decreased PRM expression and infertility in men (Kramer *et al.* 1997). Furthermore, deletion of this element in transgenic mice harboring a copy of the human PRM locus recapitulates this perturbed PRM expression (Martins *et al.* 2004). Irrespective of the above, nuclear matrix association within this region clearly differs from that observed in somatic cells (Fig. 2, Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al.* 2009). Studies of higher order chromatin structure within the orthologous domains of this transgenic model will inform the degree to which this regulation is species specific.

Conclusion

The appreciation that sperm functionally package several layers of developmentally important information has become apparent. In human sperm, the genomic landscape, though dominated by PRMs, is enriched in

histones at both promoters and exons. The presence of nucleosomes in these regions, some of which contain modified histones, is highly suggestive of subsequent epigenetic control in the embryo. Furthermore, nucleosome-associated DNA may also tether individual toroid loops to the nuclear matrix. Following fertilization, these sequences partnered with the sperm nuclear matrix may provide the zygote a platform for the transgenerational inheritance of paternal chromatin structure. These potentially inherited chromatin associations may demarcate replicons utilized in early development. Perhaps some of these events are directed by factors translated from paternally derived mRNAs. This subpopulation of RNAs, like the rest of the transcripts present in sperm, is undoubtedly delivered to the oocyte. But are these transcripts functional?

The nuclear environment of the mammalian sperm continues to yield new discoveries. Many of these will be instrumental in elucidating the mechanisms controlling the early moments following conception. However, this will require the use of non-human models. Irrespectively, male fertility biomarkers may soon emerge as local chromatin structure and/or RNA signatures continue to be developed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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Annexe 2 : Chapter 16 : Post-meiotic DNA damage and response in male germ cells, Leduc *et al.*, DNA repair, Intech (2011)

16

Post-Meiotic DNA Damage and Response in Male Germ Cells

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1. Introduction

Spermatids are haploid cells that differentiate into spermatozoa and may be considered as an interesting model of DNA damage response and repair. Key features, such a unique set of chromosomes, radioresistance to apoptosis, the presence of known end-joining DNA repair pathways and an underlying prerogative to limit the transmission of any mutation to the next generation, make them a unique cell type to provide new insights on similar pathways in somatic cells. Although DNA damage signaling and repair mechanisms have been extensively studied during meiosis, the contribution of post-meiotic germ cells to the genetic integrity of the male gamete have been overlooked. In this chapter we present clear evidences that the haploid phase of spermatogenesis, termed spermiogenesis, may represent an even greater challenge for the maintenance of the genetic integrity of the male gamete. Since transient DNA strand breaks are intrinsic to the differentiation program of spermatids (Leduc et al., 2008a; Marcon and Boissonneault, 2004), a better understanding of DNA repair pathways involved may shed some light on their potential contribution to male-driven *de novo* mutations and eventually to some unresolved cases of male infertility. This chapter will mainly focus on DNA breaks occurring in the post-meiotic phase of the spermatogenesis and how germ cells deal with it.

2. Spermatogenesis

In most mammals, testes are found in the scrotum and are maintained at lower temperature (2-8°C) than the core body (Harrison and Weiner, 1949; Setchell, 1998). In fact, spermatogenesis is known to work better at lower temperature and it was shown that fertility declines with scrotal hyperthermia. For example, higher scrotal temperature due to fever or lifestyle correlates with decreased semen quality in humans (reviewed in Jung and Schuppe, 2007).

To support germ cells in their development, Sertoli cells are located at the basal lamina, throughout the seminiferous tubules (Russell, 1990). They provide nutrients and essential

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molecules to the differentiating germ cells and regulate the seminiferous tubular fluid (Griswold, 1998; Rato et al., 2010). The Sertoli cells are interconnected by different junctions, creating a unique barrier between surrounding blood vessels and differentiating germ cells that is known as the "blood-testis barrier" (Cheng and Mruk, 2002; Dym and Fawcett, 1970; Setchell, 1969; Vogl et al., 2008). This barrier restricts molecules to enter or exit the adluminal compartment, creating a microenvironment with diverse transporters and preventing immunological response against germ cells (reviewed in Mital et al., 2011).

Spermatogenesis is the cellular differentiation pathway leading to the production of male gametes. This process takes place in seminiferous tubules in the testis, which is a unique environment regulated by follicle-stimulating and luteinizing hormones, secreted by the pituitary gland (Russell, 1990). From birth to puberty, seminiferous tubules are composed of spermatogonia, the precursor stem cell of the germinal cells, and Sertoli cells. At the onset of puberty, spermatogonia undergo mitosis and commit to the differentiation pathway leading to male germ cells. As the germ cells differentiate, they migrate towards the lumen of the tubule, creating an organized stratified structure. Spermatogenesis can be divided in two phases, spermatocytogenesis and spermiogenesis.

Spermatocytogenesis is the process by which a spermatogonium differentiates into primary spermatocytes, which duplicate their DNA to undergo meiosis and become haploid spermatids. This meiotic division is important to create genetic variations by meiotic crossovers and random segregation of parental chromosomes. Spermiogenesis is characterized by the radical metamorphosis of the haploid spermatid into spermatozoa, requiring the reorganization of their organelles. The acrosome, a cap-like structure needed for enzymatic digestion of the oocyte outer membrane, is formed from the Golgi apparatus. At the opposite nuclear pole, the flagellum begins to grow from the centrioles and mitochondria groups at the mid-piece of the emerging flagellum to produce the required energy for its later motion. Finally, the spermatid is stripped of most of its cytoplasm and ultimately released in the lumen of the seminiferous tubule. Most interestingly, the nucleus of spermatids is also remodeled and condensed to protect the paternal genome as well as providing a more hydrodynamic shape. However, this nuclear reorganization is characterized by transient DNA strand breaks that may be necessary to relieve the torsional stress as outlined below.

2.1 Chromatin remodeling process

Through the chromatin remodeling process of spermatids, the paternal genome is condensed tenfold compared to somatic cells, forming a nucleus with an hydrodynamic-shape (Balhorn et al., 1984). To achieve such a high degree of compaction, chromatin must first rely on a set of abundant transition proteins (TPs) subsequently replaced by the protamines (PRMs) (Balhorn et al., 1984; Braun, 2001). The arginine-rich PRMs bind DNA and neutralize the phosphodiester backbone of the double helix (Balhorn, 1982), allowing for a tight compaction of the DNA into torroids (Ward, 1993). Although the onset of chromatin remodeling is poorly understood, incorporation of testis-specific histone variants (Churikov et al., 2004; Govin et al., 2007; Lu et al., 2009; Martianov et al., 2005; Yan et al., 2003) and regulated post-translational modifications of histones, such as acetylation (Christensen et al., 1984; Grimes and Henderson, 1984; Marcon and Boissonneault, 2004; Meistrich et al., 1992), ubiquitination (Baarends et al., 1999; Chen et al., 1998), methylation (Godmann et al., 2007; van der Heijden et al., 2006) and phosphorylation (Blanco-Rodríguez,

2009; Krishnamoorthy et al., 2006; Leduc et al., 2008a; Meyer-Ficca et al., 2005) are known to initiate and participate in the exchange from histones to the more basic proteins such as TPs and PRMs during the transition from the round to the elongated spermatids. These modifications are known to modulate the affinity of histones for DNA, but also the affinity of other proteins for histones, such as chromatin remodelers, DNA repair proteins or the transcription machinery. After spermiation, this unique protamine-based chromatin is further stabilized by the creation of intraprotamine disulfide bonds during the transit through the epididymis (Golan et al., 1996). Therefore, protamination provides both chemical and mechanical protection to the male haploid genome. Interestingly, protamination of the male genome is not complete and varies across species. In the mouse spermatozoon, about 1-2% of the genome remains organized by histones (Balhorn et al., 1977), whereas up to 15% of histones are still found in humans spermatozoa (Gatewood et al., 1990; Gusse et al., 1986; Tanphaichitr et al., 1978). This observation lead to hypothesize that these nucleosomes could serve as epigenetic markers for embryonic development (Arpanahi et al., 2009; Zalenskaya et al., 2000) (for a more detailed review on the sperm chromatin organization, see Johnson et al., 2011)

3. Nature of endogenous DNA damages during spermiogenesis

3.1 Single strand damage and repair

Depending on the type of damage, specific pathways achieve single strand damage repair (see Table 1). Mispaired DNA bases that primarily arise during replication are corrected by mismatch repair (MMR), while small chemical alterations of DNA bases such as alkylation, deamination and oxidative damage are repaired by base excision repair (BER) (Mukherjee et al., 2010; Robertson et al., 2009). More complex lesions such as those induced by UV (pyrimidine dimers and helix-distorting lesions) are corrected by nucleotide excision repair (NER), a multistep pathway that involves more than 30 proteins (Hoeijmakers, 2009; Nouspikel, 2009). DNA nicks are repaired by single-strand break repair (SSBR). These DNA repair pathways are known to be present and active during spermiogenesis (Olsen et al., 2001; Schultz et al., 2003). To our knowledge, single-strand damages do not present a major threat to spermatids. With the exception of exposures to toxicant that could challenge these pathways, in normal conditions, single-strand DNA damage during spermiogenesis is likely attributed to the massive transcription that is taking place at these steps and is efficiently resolved by spermatids (Olsen et al., 2001). DNA double-strand breaks were reported as part of the normal differentiation program of spermatids during spermiogenesis which may represent an important source of genetic instability and therefore we will focus on these pathways.

3.2 Double-strand breaks in spermatids

3.2.1 Possible origin of DNA breaks

Several hypotheses have been formulated to elucidate the origin and role of DNA strand breaks in spermatids. Sakkas and colleagues suggested that "abortive apoptosis" may be the cause since abnormal human spermatozoa presented some apoptotic-like features (Sakkas et al., 1999). Further investigation led to the demonstration that other biomarkers of apoptosis in sperm cells were present such as BCL-X, TP53, caspases, in addition to diverse structural defects (Baccetti et al., 1997; Donnelly et al., 2000; Gandini et al., 2000; Sakkas et al., 2002; Weng et al., 2002). Due to technical limitations at the time, DNA breaks were only observed

DNA repair pathways		DNA damages	Implicated proteins
Mismatch repair (MMR)		Mispaired DNA bases	MSH1-6, MLH1, MLH3, PMS1, PMS2, EXO1, RPA, PCNA, RFC
Base excision repair (BER)	Short-patch	Small DNA bases chemical alteration arising from alkylation, deamination and oxidative damage	UNG, APEX1, POL β, XRCC1, LIG3
	Long-patch		UNG, APEX1, POL β / δ, FEN1, PCNA, LIG1
Nucleotide excision repair (NER)	Transcription -coupled or not	Pyrimidine dimer	XPC complex, DDB complex, ERCC3 (TFIIH), XPA-RPA complex, ERCC5 (XPG), ERCC1-ERCC4 (XPF), LIG3, DNA polymerase δ
Single strand break repair (SSBR)	Short-patch or long-patch	Single strand break (SSB)	APE1, PNKP, APTX, TDP1, POL β/δ/ε, PCNA, XRCC1, LIG1/3, FENI, PARP

Table 1. Summary of the single strand DNA repair pathways in mammalian cells (Ciccia and Elledge, 2010; Hoeijmakers, 2009; Martin et al., 2010; Mukherjee et al., 2010; Nouspikel, 2009; Robertson et al., 2009).

in a subset of the whole population of elongating spermatids and therefore abortive apoptosis could represent a sound explanation. However, some studies demonstrated that round spermatids are radioresistant to apoptosis and may not have the proper machinery and checkpoints to trigger such process (Ahmed et al., 2010; Oakberg and Diminno, 1960). Furthermore, our group have demonstrated that transient DNA breaks were present in the whole population of elongating spermatids of fertile mice and humans during chromatin remodeling and were therefore part of the normal differentiation program of these cells (Marcon and Boissonneault, 2004). The persistence of these breaks beyond the chromatin remodeling steps in pathological conditions may explain the presence of DNA fragmentation found in spermatozoa of infertile men (Leduc et al., 2008b).

Generation of controlled DNA breaks either single- or double-stranded may be important to relieve the torsional stress induced by the withdrawal of histones (Boissonneault, 2002). The simple mechanical stress resulting from the accumulation of free supercoils could induce non-B DNA structures and possibly DNA breaks as the chromatin remodeling is extensive and takes place within many differentiation steps. However, enzymatic induction of DNA strand breaks is more likely, as their free ends can be end-labeled with polymerases that require a 3'OH as substrate, such as the terminal deoxynucleotidyl transferase (TdT) used in TUNEL labeling. Specific nucleases could be involved in this process, and it is not excluded that retrotransposon nucleases could play a role as they are expressed throughout

spermatogenesis, including in the nucleus of spermatids (Branciforte and Martin, 1994; Ergün et al., 2004; Gasior et al., 2006). However, topoisomerases have long been considered likely candidates to support chromatin remodeling from bulky histone-bound chromatin to compact and transcriptionally inert protamine-bound DNA because of their ubiquitous role in chromosome dynamics during the somatic cell cycle (McPherson and Longo, 1993).

3.2.2 Topoisomerases as candidates to supercoiling removal

Change in DNA topology can be achieved by single-strand breaks (SSBs) generated by type I topoisomerase, which modifies the linking number in steps of one. Single-strand breaks would be considered a much smaller threat for the genome's integrity of spermatids than a DSB that could be generated by type II topoisomerases. However, chromatin remodeling in spermatids was clearly shown to be associated with an increase in type II topoisomerase (Chen and Longo, 1996; Laberge and Boissonneault, 2005; Leduc et al., 2008a; McPherson and Longo, 1992, 1993; Meyer-Ficca et al., 2011b; Roca and Mezquita, 1989). A possible link between type II topoisomerases and DNA breaks found in elongating spermatids was suggested by the elimination of DNA breaks in spermatids nuclei incubated with type II topoisomerase inhibitors such as suramin and etoposide (Laberge and Boissonneault, 2005). In mammal cells, the α and β isoforms of topoisomerase share more than 80% of homology and are differentially expressed. Topoisomerase IIα (TOP2A) is mostly found in replicating cells whereas topoisomerase IIβ (TOP2B) predominates in quiescent cells (Morse-Gaudio and Risley, 1994; Turley et al., 1997). Using immunofluorescence on mouse testis sections, we have observed TOP2B foci in nuclei of elongating spermatids whereas TOP2A remained undetected in these cells but highly present in spermatocytes (see Figure 1) (Leduc et al., 2008a). Detection of TOP2B in elongating spermatids is not surprising, as spermatids are non-replicative cells. Recent studies confirmed the involvement of TOP2B in elongating spermatids (Meyer-Ficca et al., 2011b) and also observed its presence further downstream of the male germ cells differentiation program as part of the nuclear matrix of sperm cells, supporting its earlier role in the chromatin remodeling of spermatids (Shaman et al., 2006).

3.2.3 Topoisomerases and DNA repair

Type II topoisomerase activity may be modulated by post-translational modifications, such as phosphorylation by kinases and poly (ADP-ribosyl)ation by poly (ADP-ribose) polymerases (PARPs), a well-known family of proteins involved in a multitude of nuclear events, such as DNA repair and chromatin remodeling. This complementary interaction between TOP2B and PARPs may be involved in numerous cellular processes. For example, TOP2B and PARP1 are known to modulate transcription in somatic cells (Ju et al., 2006). Furthermore, these proteins may be important constituents of the nuclear matrix; Zaalishvili and coworkers observed the stimulation of cleavage of nuclear matrix associated DNA loops of neuron and leukocyte nucleoids when incubated in buffer supporting topoisomerase and PARP activity (Zaalishvili et al., 2005). This stimulation was reversed by the addition of thymidine, a PARP inhibitor. The authors suggested that a PARP-modified topoisomerase II may cut efficiently but the (ADP-ribosyl)ation could inhibit the religation. Recently, Meyer-Ficca and colleagues demonstrated a possible modulation of TOP2B activity by PARP and PARG *in vitro* using recombinant proteins as well as *in vivo* during mouse spermiogenesis through the use of inhibitors and knockout mouse models (Meyer-Ficca et al., 2011b). According to their findings, there is a functional relationship between the DNA strand break activity of TOP2B and the DNA strand break-dependent activation of

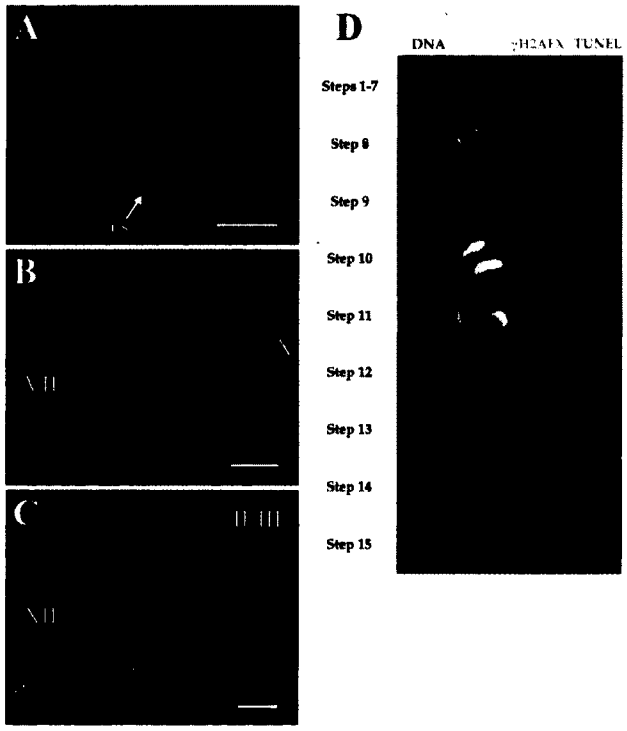

Fig. 1. Presence of type II topoisomerases, hyperacetylated histone H4, γH2AFX and DNA breaks during mouse spermiogenesis. (A) Overlay of TOP2B immunofluorescence (green) and DAPI nuclear staining (blue) of a stage IX tubule demonstrating the presence of TOP2B in nuclei of elongating spermatids (ES) at the onset of chromatin remodeling. (B) Overlay of TOP2B immunofluorescence (green) and DAPI (blue) nuclear staining of stages VII and X tubules. (C) Overlay of immunofluorescence of TOP2A (green) and TO-PRO3 (red) nuclear staining of stages XII and II-III demonstrating the nuclear presence of TOP2A in zygotene and pachytene spermatocytes but complete absence in spermatids. (D) Detection of hyperacetylated histone H4 and γH2AFX by immunofluorescence and DNA breaks by TUNEL during mouse spermiogenesis. DNA was counterstained by TO-PRO3. (A-C) Immunofluoresence on Bouin-fixed testis sections was done as previously described(Leduc et al., 2008a). (D) Squash preparation were done as previously described (Kotaja et al., 2004; Leduc et al., 2008a), fixed with ice-cold ethanol and processed for TUNEL and immunofluorescence. Bars = 10 μm (A and B), 20 μm (C) and 5μm (D).

PARP enzymes. Moreover, alteration in the PAR metabolism leads to a greater retention of histones in spermatozoa (Meyer-Ficca et al., 2011a). Whether PARP1 is involved directly in chromatin remodeling, DNA repair or combination of both in spermatids remains to be determined and will be discussed further in section 4.

4. DNA damage response and DNA repair of double-strand breaks

4.1 DNA damage signaling pathways

The first step following a DSB is the detection of the DNA damage by sensors (Lamarche et al., 2010). At least four independent sensors can detect DSBs: PARPs in all cases of SSBs and, to a lesser extent, DSBs, Ku70/80 in D-NHEJ, MRE11-RAD50-NBS1 (MRN) complex in all cases of DSBs and replication protein A1 (RPA) in HR (Ciccia and Elledge, 2010; Lamarche et al., 2010).

As previously stated in section 3.2.3, the presence and activity of PARP1 and PARP2 have been recently investigated during spermiogenesis of mouse and rat (Ahmed et al., 2010; Dantzer et al., 2006; Meyer-Ficca et al., 2005; Meyer-Ficca et al., 2011a; Meyer-Ficca et al., 2009; Meyer-Ficca et al., 2011b). Although the individual absence of these proteins leads only to subfertility in male, it is believed that they play a key role in the maintenance of genomic integrity of spermatids. As discussed previously, PARPs may be involved in DNA repair and signaling, in the drastic chromatin remodeling of spermatids and even in the repackaging of their genome with protamines (Quénet et al., 2009). However, the embryonic lethal phenotype of double knockout mouse prevent a better assessment of their critical role during spermiogenesis, as the absence of one can be compensated for by the other. The Ku heterodimer binds to DSB ends and is required for the non-homologous end-joining pathway (NHEJ). In addition to its role in DNA repair, Ku proteins are also required for the maintenance of telomeres and subtelomeric gene silencing (Celli et al., 2006; Lamarche et al., 2010). KU70 is present during the spermiogenesis of mouse (Goedecke et al., 1999; Hamer et al., 2003), human (Leduc *et al.*, unpublished observations), and grasshoppers (Cabrero et al., 2007), but seems to decrease as spermiogenesis proceeds, most notably after the expulsion of histones. Although initial analyses of the implication of MRN complex as sensor in non-homologous end-joining pathways produced conflicting results (Di Virgilio and Gautier, 2005; Huang and Dynan, 2002), recent studies showed that siRNA mediated knockdown of Mre11 results in reduced end-joining efficiency in both D-NHEJ and B-NHEJ pathways (Rass et al., 2009; Xie et al., 2009) and should be considered a good candidate for DNA breaks detection and signaling in spermatids. As for Ku proteins, Mre11 is also present during spermiogenesis (Goedecke et al., 1999). Contrary to these DNA break sensor proteins, RPA may not play such an important role during spermiogenesis as spermatids, being haploid, cannot rely on HR repair processes.

The detection of DNA damage by sensors activates proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family such as ATM, ATR, and DNA PKcs and members of the PARP family. These proteins post-translationnally modify key protein targets triggering a signal transduction cascades that forms the DNA damage response (DDR) (Lamarche et al., 2010). During mouse spermiogenesis, ATM and DNA PKcs are present and active (Ahmed et al., 2010; Scherthan et al., 2000). These kinases are responsible for the phosphorylation of the histone H2A variant, H2AFX, at serine 139 (γH2AFX, previously referred to as γH2AX), which quickly occurs after a DSB. This modification can spread up to a 2 Mbp region flanking all DSBs (Kinner et al., 2008) and it could help the recruitment of other proteins of the DDR (Celeste et al., 2003). Within minutes following DNA damage, γH2AFX appears at discrete nuclear foci that dissolve after the completion of DNA repair. It remains unclear whether γH2AFX is replaced completely with new H2AFX histones, or simply dephosphorylated, but strong evidences suggest that the latter mechanism is

prominent (Chowdhury et al., 2005; Rogakou et al., 1999). Therefore, the implication of γH2AFX in all cases of DSBs makes it a novel biomarker for DSBs detection by immunoflorescence (Mah et al., 2010; Mah et al., 2011). Upon γH2AFX signaling, specific pathways are recruited according to cell type or the cell cycle phase (Shrivastav et al., 2008). The presence of γH2AFX during spermiogenesis has been first shown in rats (Meyer-Ficca et al., 2005) and we confirmed its presence at the corresponding steps during mouse spermiogenesis (Leduc et al., 2008a) (see Figure 1). As shown in Figure 1, the presence of γH2AFX and hyperacetylated histone H4, a biomarker of chromatin remodeling coincides with the presence of TOP2B. These results confirm the previously published strong TUNEL labeling of elongating spermatids during chromatin remodeling (Laberge and Boissonneault, 2005; Marcon and Boissonneault, 2004).

Therefore, spermatids undergo multiple transient DSBs, inducing a classic DDR signaling. In addition, as seen by immunofluorescence in Figure 1, γH2AFX is present in all spermatids throughout chromatin remodeling as part of the normal process of maturation of spermatids. The pattern of γH2AFX in spermatids as seen in Figures 1 and 2 is dependent on fixation and tissue processing; ethanol fixation provides a better context for TUNEL labeling but alters nuclear distribution of proteins. Furthermore, we have also found the presence of γH2AFX and DNA breaks during human spermiogenesis (see Figure 2), while other groups subsequently demonstrated similar DDR signaling in grasshoppers (Cabrero et al., 2007) and even in the algae *Charas vulgaris* (Wojtczak et al., 2008). Moreover, the presence of DNA breaks has also been found during spermiogenesis of drosophila (Rathke et al., 2007). Altogether, these results suggest that the DDR triggered by endogenous breaks in spermatids is evolutionary conserved and could represent a new source of male-driven genetic instability in species where gametogenesis requires condensation of the genetic material.

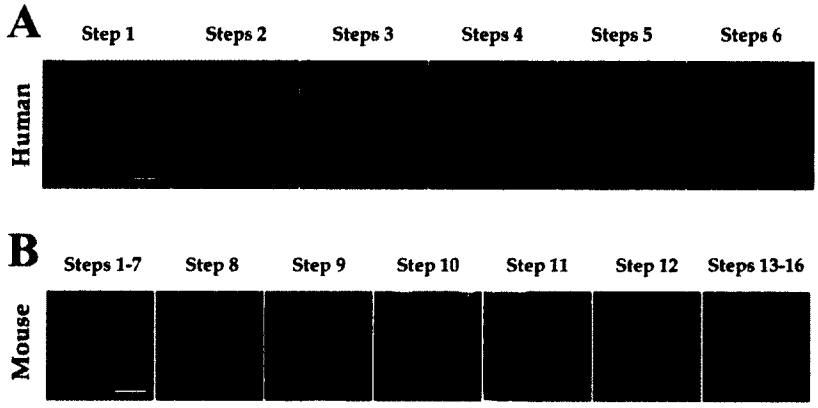

Fig. 2. Detection of γH2AFX during spermiogenesis of human (upper panel), and mouse (lower panel). DNA was counterstained by TO-PRO3. Bars = 5μm. Immunofluoresence on paraformaldehyde-fixed testis sections was done as previously described (Leduc et al., 2008a).

4.2 Evidences of an active DNA repair system during spermiogenesis

Although these DSBs are considered the most harmful genetic damage for a cell, we know from experimental data (Marcon and Boissonneault, 2004) that these breaks are repaired by the end of spermiogenesis in fertile animals. The disappearance of γH2AFX in mouse spermatids (step 13 to 16) shown in Figure 1 cannot be associated with completion of DNA repair or dephosphorylation as a majority of histones are expulsed from the nucleus to be replaced by PRMs. However, we obtained other evidences of an active DNA repair system at these steps by demonstrating incorporation of dNTPs *in situ* that is sustained through all the chromatin remodeling steps (see Figure 3) (Leduc et al., 2008a). Furthermore, as seen in Figure 1, the appearance and disappearance of TUNEL labeling is coincident with γH2AFX fluorescence. To confirm that the loss of TUNEL labeling was associated with DNA repair and not with the lack of penetrability of the TdT in the nuclei of condensed spermatids, we decondensed spermatids prior to TUNEL with similar results (Marcon and Boissonneault, 2004) (Acteau et al., unpublished observations). Therefore, DNA breaks are properly repaired by the end of the spermatids differentiation program. As previously stated, mammalian cells can rely on four DNA DSBs repair pathways, each of which having different degree of fidelity. As spermatids differentiate to spermatozoa with fertilizing potential, any errors due to faulty or incomplete DNA repair may be transmitted to the next generation. Severe alteration in the repair process may cause infertility or possibly be incompatible with embryonic development (Leduc et al., 2008b).

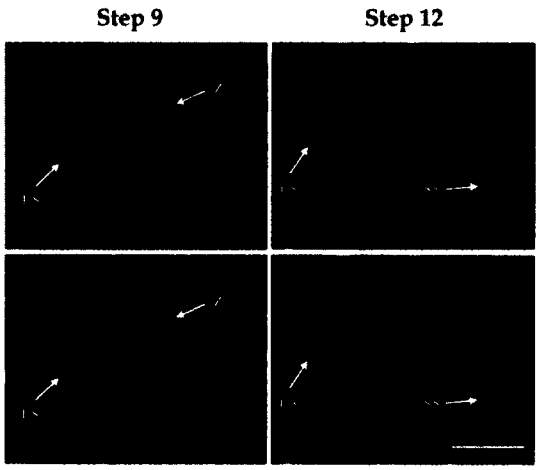

Fig. 3. In situ endogenous DNA polymerase activity assay (Leduc et al., 2008a) on squash preparation of stage IX and XII tubules. DNA was counterstained by DAPI. Bar = 10 µm

4.3 Towards identification of DNA repair pathways

Double-strand breaks are processed either by homologous recombination, single-strand annealing (SSA) or non-homologous end-joining (Caldecott, 2008). Two types of NHEJ are available to mammalian cells: the pathway that is dependent of DNA PKcs (referred to as D-

NHEJ) and the alternative (or "back-up") pathway (referred to as B-NHEJ), which is also known as microhomology-mediated end-joining (MMEJ) (Ciccia and Elledge, 2010; West, 2003). Therefore, we will discuss known somatic DNA repair pathways and their potential role in spermatids when supported by published data.

4.3.1 Homologous recombination and single-strand annealing

Given the haploid character spermatids, HR could not take place as sister chromatids or homologous chromosomes are not available for recombination. Considering that HR precisely restores the genomic sequence of the broken DNA ends by utilizing sister chromatids as template for DNA repair, HR usually occurs in late S2 and G phase of the cycle in mammals (Kass and Jasin, 2010), whereas spermatids are considered to be in a G1-like phase (Ahmed et al., 2010). Upon resection at DNA breaks by the MRN complex, two different pathways are usually possible: HR or SSA (Wold, 1997). The SSA pathway could use repetitive DNA sequences to promote the DNA repair of DSBs in spermatids (Hartlerode and Scully, 2009; Motycka et al., 2004). This pathway is known to introduce errors such as deletions, insertions and even be a substrate for chromosomal translocations (Griffin and Thacker, 2004). There is currently no evidence that spermatids use SSA rather than NHEJ to repair DSBs, but some key proteins of this pathway, although also part of the NER pathway (see Table 1 and Table 2), are present during spermiogenesis including ERCC1 (Hsia et al., 2003; Paul et al., 2007) as well as XPF (Shannon, 1999).

DNA repair pathways	Implicated proteins	Typical error
Homologous recombination (HR)	BRCA1/2, Rad51, XRCC2, LIG1, RPA	Error free
Single strand annealing (SSA)	ERCC1, ERCC4 (XPF), Rad52	Indels (++) Chromosomal translocation (++)
Non-homologous end-joining DNA PK dependant (D-NHEJ)	DNA PKcs, Ku70, ARTEMIS, XRCC4, LIG4, XLF (NHEJ1)	Indels (+) Chromosomal translocation (+)
Alternative non-homologous end joining (B-NHEJ)	PARP, XRCC1, LIG3	Indels (+) Chromosomal translocation (+)

Table 2. DNA double-strand break repair pathways and their typical error. (+) Occasional, (++) frequent (Ciccia and Elledge, 2010; Griffin and Thacker, 2004).

4.3.2 Non-homologous end joining

Besides SSA, B-NHEJ and D-NHEJ are potentially available for the repair of double-strand breaks during spermiogenesis (Leduc et al., 2008a; Leduc et al., 2008b). In somatic cells, NHEJ pathways promote the religation of DSBs, introducing small insertions and deletions. NHEJ pathways operates throughout the cell cycle but are most active during G1 phase

because HR cannot proceed during that time (Daley et al., 2005). Spermatids provide a similar cellular context as G1 phase of somatic cells. However, dynamics of DNA repair by NHEJ pathways, as illustrated in irradiated round spermatids, are much slower (Ahmed et al., 2010). According to Ahmed and colleagues both pathways are present and active during mouse spermiogenesis: spermatids of SCID mice, lacking the D-NHEJ because of the absence of DNA PKcs, displayed slower repair than those from wild type mice (Ahmed et al., 2010). Further studies on the end-joining pathways in elongating spermatids will be required as these are known to be error-prone in somatic cells. This may also be the case in spermatids. Although an attenuation of the frequency of mutations may be found in the germ line (Walter et al., 1998), the chromatin remodeling in spermatids may still be the key differentiation steps where most of the new mutations repertoire is being produced for the transmission to the next generation.

5. Possible consequences on fertility and genetic integrity

5.1 Incomplete DNA repair

High level of sperm DNA fragmentation, sperm DNA damages and chromatin alterations decrease pregnancy rates in natural fertilization, intrauterine insemination and *in vitro* fertilization (Bungum et al., 2007; Duran, 2002; Evenson et al., 1999; Evenson and Wixon, 2006; Spano et al., 2000; Zini, 2011). Moreover, pregnancy loss following *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatments has also been attributed to poor sperm DNA integrity (Zini et al., 2008). Although sperm DNA fragmentation is more frequent in infertile men, sperm of fertile men display DNA fragmentation but to a lesser extent (Bellver et al., 2010; Brahem et al., 2011; Perrin et al., 2009; Rybar et al., 2009; Venkatesh et al., 2011; Watanabe et al., 2011). After fertilization, the oocyte can efficiently repair some paternal DNA damages (Brandriff and Pedersen, 1981; Marchetti et al., 2007), but in the case of highly damaged sperm DNA, this could exceed the DNA repair capacity of the oocyte leading to some genetic aberrations, developmental arrest or pregnancy loss.

5.2 Faulty repair

Structural aberrations

Chromosomal structural aberrations such as translocations, deletions and inversions, may originate from meiotic recombination involving non-allelic repeated DNA sequences (Heyer et al., 2010). However, since about 80% of chromosomal rearrangements are reported to be of paternal origin (Buwe et al., 2005; Thomas et al., 2006) and that male and female meiosis involves similar genetic mechanisms (Gu et al., 2008; Thomas et al., 2006), one can surmise that yet another process unique to male gametogenesis may be involved. We therefore hypothesize that the chromatin remodeling process in spermatids, generating transient double-strand breaks, may provide the proper context for faulty repair and induction of transgenerational polymorphism. In addition, it is tempting to speculate that, because chromatin condensation occurs, free DNA ends are brought in juxtaposition, increasing the chance of NHEJ repair involving two different chromosomes, which may lead to translocations. Interestingly, chromosomes possess their own territory within the nucleus of somatic cells and in sperm cells (Hazzouri et al., 2000; Manvelyan et al., 2008; Mudrak et al., 2005; Zalenskaya et al., 2000). Moreover, chromosomes known to have higher translocation rates have close chromosomal territories in somatic cells (Branco and Pombo, 2006; Brianna

Caddle et al., 2007; Manoj S Gandhi and Nikiforov, 2009). Thus, chromosomes with close chromosomal territories in spermatids could be more prone to interchromosomal translocation during chromatin remodeling. In addition, the potential for the spermatidal chromatin remodeling to produce non-B DNA structure may exacerbate the propensity for spermatids to produce translocation (Hidehito Inagaki and Kurahashi, 2009; Raghavan and Lieber, 2004). Further investigation is needed on the mechanism and potential involvement of chromatin remodeling in such events.

Insertions and deletions

As outlined above, NHEJ repair pathways are known to create insertions and deletions (indels) as they use microhomology to join the two DNA ends. This type of mutations may be particularly harmful in coding sequences as it may cause codon frameshifts or alteration of mRNA splicing. Moreover, Y chromosomes microdeletions, associated with increased male infertility, may exhibit the classical signature of micro-homology based DNA repair pathways such as SSA and B-NHEJ as the deletion occurs between repetitive, often palindromic sequences (Paulo Navarro-Costa and Plancha; Yen, 1998). Although SSA is available during most of spermatogenesis, the B-NHEJ signature on the highly repetitive Y chromosome may be indicative of a faulty DNA repair in spermatids as this pathway is inhibited during meiosis.

Dynamic mutations

Several diseases with dynamic mutation, characterized by the expansion over generation of a trinucleotidic repeat (TNRs), are associated with a paternal bias of expansion, such as Huntington disease (HD), spinocerebellar ataxia type 2 and 7 (Cancel et al., 1997; Stevanin et al., 1998; Zühlke et al., 1993). TNRs are microsatellites sequences in coding or non-coding region of the genome. Their stability, which is relative to the chance of adopting a secondary structure, is dependent of the nature of the sequence and the length of the TNR (Kovtun et al.; Tóth et al., 2000). The exact mechanism of TNR expansion or contraction is still not clear. However, studies show strong evidences that TNR expansion in the huntingtin gene occurs during spermiogenesis; in a transgenic mouse model carrying the mutated human gene, an increased length of the CAG repetition was observed in mature spermatozoa but not in early haploid spermatids and other tissues. Kovtun and McMurray also demonstrated the involvement of MSH2, a protein involved in the gap repair and mismatch repair pathways, as this expansion was absent in HD mice MSH2-/- (Albin and Tagle, 1995; Kovtun and McMurray, 2001). The remodeling chromatin of spermatids may promote secondary structure formation at TNRs, providing an ideal context for such mutations.

6. Conclusion

The chromatin remodeling in spermatids involves transient DNA-strand breaks. Our group has generated evidences that a significant number of double-strand breaks are generated. These DSBs seemingly trigger a damage response as H2AFX is phosphorylated and a DNA repair pathway yet to be identified. As a result, no such DSBs are found in the mature sperm unless a pathological condition prevails. The non-templated DNA repair of these transient DSBs are expected to introduce small mutations likely distributed randomly across the haploid genome although their distribution remains to be established. Meiosis is well known to produce new combination of alleles but is not a primary driver of sequence divergence (Noor, 2008). Potential new gene function must arise through point mutations or

indels and the present review suggests chromatin remodeling of spermatids as an appropriate context for such induction of new polymorphism and possible translocations. Although the frequency of mutation may be lower in germ cells than in somatic cells (Walter et al., 1998), we hypothesize that most of the new mutations generated during spermatogenesis may be through the process of endogenous strand breaks and repair during spermiogenesis. Owing to the 1% chance for a random mutation to occur within genes due to exonic representation in the genome, most mutations are expected to be silent but, if within coding sequences, potential alteration of gene function may be transmitted. In summary, repair of the endogenous DSBs in spermatids may represent a new male-driven source of genetic variation.

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Annexe 3 : Spermiogenesis and DNA repair : a possible etiology of human infertility and genetic dirorders, Leduc *et al.*, Systems Biology in Reproductive Medicine (2008)

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Hypothesis

Spermiogenesis and DNA Repair: A Possible Etiology of Human Infertility and Genetic Disorders

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This paper reviews the possible origin of sperm DNA fragmentation and focuses on the nuclear events associated with spermiogenesis as a potential source of genetic instability and reduced fertilizing potential of the mature male gamete. Recent findings suggest a programmed DNA fragmentation and DNA damage response during the chromatin remodeling steps in spermatids. We also discuss the spermatid DNA repair mechanisms and the possible involvement of condensing proteins, such as transition proteins and protamines, in the process, as this DNA fragmentation is normally not found in late spermatids. We propose that alterations in the chromatin remodeling steps or DNA repair in elongating spermatids may lead to persistent DNA breaks. This vulnerable step of spermiogenesis may provide a clue to the etiology of sperm DNA fragmentation associated with infertility in humans. This vulnerability is further emphasized given the haploid character of spermatids that must resolve programmed double-stranded breaks by an error-prone DNA repair mechanism. Therefore, spermiogenesis has probably been overlooked as an important source of genetic instability.

KEYWORDS chromatin, DNA repair, infertility, spermiogenesis, transition proteins

Abbreviations: AcH4: Hyperacetylated histone H4; ART: Assisted reproduction technology; SCSA: sperm chromatin structure assay; BER: Base excision repair; DDR: DNA damage response; DSB: Double-strand breaks; ES: Elongating spermatid; ICSI: Intracytoplasmic sperm injection; MMR: Mismatch repair; NHEJ: Non-homologous end joining; ROS: Reactive oxygen species; SCSA: Sperm chromatin structure assay; TDP1: Tyrosyl-DNA phosphodiesterase 1; TP: Transition protein; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling; HR: homologous recombination; TOP2B, topoIIβ: topoisomerase IIβ.

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INTRODUCTION

Male infertility is often associated with a relatively high DNA fragmentation level and an altered or incomplete chromatin packaging in mature spermatozoa [Fatehi et al. 2006; Irvine et al. 2000; Muratori et al. 2000]. In fact, DNA fragmentation is apparently linked to abnormal chromatin packaging in mature sperm [Gorczyca et al. 1993; Manicardi et al. 1995]. Assessment of sperm chromatin structure has been shown to have a good predictive value in the assessment of human fertility over the classical parameters recommended by WHO [Virro et al. 2004]. Alterations in the nuclear integrity of the male gametes have also been associated with

de novo genetic disorders, developmental and morphological defects, cancer and miscarriage [Emery and Carrell 2006; Kleinhaus et al. 2006; Marchetti and Wyrobek 2005; Tesarik et al. 2004]. Sperm DNA fragmentation can also compromise ART (assisted reproduction technology) [Tesarik et al. 2004].

New tools were developed to assess the nuclear integrity of spermatozoa [for a review, see Evenson and Wixon 2006]. These include the sperm chromatin structure assay (SCSA) and the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) a sensitive tool to label DNA at strand breaks. The origin of DNA fragmentation in human spermatozoa remains unknown and multiple sources have been proposed including abortive apoptosis, abnormal chromatin packaging, generation of reactive oxygen species and premature release of spermatids from Sertoli cells [Muratori et al. 2006; Oliva 2006; Sakkas et al. 2002; Tesarik et al. 2006]. We have also proposed that the DNA fragmentation detected in mature spermatozoa may originate from the persistence of the transient DNA strand breaks observed at mid-spermiogenesis steps [Laberge and Boissonneault 2005a]. These transient DNA strand breaks appear to be part of the normal differentiation program of the spermatids and are produced to either support the important change in DNA topology or as a result of a transient exposure to endogenous nuclease during the chromatin remodeling.

Although male infertility is affected by a variety of environmental, behavioral and genetic factors, resulting in the impairment of spermatogenesis at various levels [Toshimori et al. 2004], the present review focuses on the nuclear events associated with spermiogenesis as a potential source of genetic instability and reduced fertilizing potential of the mature male gamete.

ABORTIVE APOPTOSIS

During spermatogenesis, apoptosis is essential for balancing the Sertoli cell/germ cell ratio. Sakkas and colleagues previously reported the high occurrence of the cell surface protein Fas, in the ejaculates from patients with abnormal semen parameters [Sakkas et al. 1999]. Apoptosis-like features other than the Fas ligand have also been observed in sperm including ultrastructural defects such as irregular, enlarged or fragmented nuclei with fragmented or marginated chromatin, cytoplasms with aggregated organelles, alteration in mitochondrial integrity, the presence of Bcl-x, caspases and p53 [Baccetti et al. 1997; Donnelly et al. 2000; Gandini et al. 2000; Sakkas et al. 2002; Weng et al. 2002]. Clear evidence linking DNA fragmentation as shown by TUNEL to these features has not been presented [Muratori et al. 2006].

It is becoming clear that TUNEL positivity may not be considered as a specific marker of apoptosis. The origin of sperm DNA fragmentation, determined by this technique, cannot be related to programmed cell death. During spermatogenesis, all meiotic cells demonstrate TUNEL positivity likely because of chromosomal crossover events that necessitate double-strand breaks [Marcon and Boissonneault 2004].

REACTIVE OXYGEN SPECIES

The presence of reactive oxygen species (ROS) is associated with a loss of sperm motility and fertilizing potential, but also with DNA damage [Aitken and Krausz 2001]. Specific ROS-induced damage include loss of sperm motility, often caused by the retention of residual cytoplasm around the midpiece, and alteration of plasma membrane compromising membrane fusion events associated with fertilization. ROS-induced DNA damage was observed in ejaculates of infertile men, reflected by the measurements of 8-hydroxydeoxyguanosine (8-OhdG) [Irvine et al. 2000]. Secretion of antioxidant molecules or enzymes protects sperm cells against ROS in reproductive tracts [O et al. 2006]. ROS-induced DNA damage may arise through the imbalance between the endogenous or xenobiotics-induced ROS generation and the natural protection provided by the accessory sex glands. Moreover, use of antioxidants has led to some relative protection of human sperm DNA fragmentation [reviewed by Agarwal et al. 2004] and improved ICSI outcome [Greco et al. 2005].

Human spermatozoa are potentially more sensitive to ROS because of their relatively high residual content of histones reaching about 10 to 20 percent [Dadoune 2003; Wykes and Krawetz 2003] compared to animal models where about 1% of mouse sperm DNA remains bound to histones [van der Heijden et al. 2006]. On an evolutionary basis, this may indicate an elevated transmission of epigenetic marks to offspring but this may produce a more

vulnerable genome. Protamination protects DNA from many insults as it creates an almost crystal-like chromatin structure, making it almost inaccessible to nucleases and mutagens [Oliva 2006]. The change in DNA topology during the chromatin remodeling steps may, by itself, account for decreased sensitivity to DNA damage as the linear, non-supercoiled DNA of the condensed spermatid may be less vulnerable to damaging agents. One may hypothesize that the arginine-rich protamines, once bound to DNA, may chemically protect spermatozoa from ROS damage as polycationic molecules (polyamines) are known as free radical scavengers [Chattopadhyay et al. 2003; Chattopadhyay et al. 2006; von Deutsch et al. 2005]. Sotolongo and colleagues [Sotolongo et al. 2003] proposed a model of sperm chromatin organization by protamines where sperm DNA is condensed by forming toroids, which consist of approximately 50 kilobases attached to the nuclear matrix by a nuclease-sensitive linker. These observations strongly suggest that a limited portion of the genomic DNA of mature sperm is sensitive to nucleases or other genetic insults. Therefore, one can assume that the packaging quality may influence the contribution of ROS to DNA fragmentation.

INCOMPLETE PACKAGING

Post-translational Modifications

Striking chromatin reconstruction takes place during spermiogenesis. Although some differences have been observed among mammals [Dadoune 2003; Oliva 2006], chromatin remodeling relies on a conserved sequence of events, that is, the incorporation of histone variants and covalent modifications of nucleosomal histones, such as acetylation and ubiquitination, the displacement of most histones by transition proteins (TPs), followed by the final deposition of protamines. Although little is known about this process, one may surmise that any alteration in this nuclear sequence of events is likely to have significant consequences on the genetic integrity of the gamete. Human homologues of RAD6, a yeast ubiquitin-conjugating enzyme, have been implicated in post-replication repair and damage-induced mutagenesis. In *HR6B*-null mice, spermiogenesis is not arrested but more than 90% of spermatozoa show aberrant head morphology with deformated flagella, suggesting its role in post-meiotic chromatin remodeling [Roest et al. 1996]. Decreased acetylation of histone H4 is correlated with infertility [Sonnack et al. 2002]. Moreover, hyperacetylation seems to be related to the control of transient DNA fragmentation [Bird et al. 2002; Laberge and Boissonneault 2005b; Marcon and Boissonneault 2004; Martínez-López et al. 2001]. The association of histone hyperacetylation with an increase in DNA fragmentation is not surprising as previous reports indicate that this post-translational modification of histone H4 in somatic cells enhances sensitivity to endonucleases [Martínez-López et al. 2001].

TRANSITION PROTEINS

The role of TPs and their structural properties are not well understood, but they play an important role in the condensation of the spermatid nucleus, as mice with deletions of both major transition proteins, TP1 and TP2, are infertile [Shirley et al. 2004; Zhao et al. 2004]. As demonstrated by Shirley and colleagues [Shirley et al. 2004], the interplay between TPs and protamines is quite complex. Although a decrease in the total TP content has a direct effect on fertility, the absence of one can be compensated by the increased expression of the other, suggesting that TPs have redundant functions. However, mutants with two copies of *Tnp1* but no *Tnp2* (producing 35% of normal TP1 level) have severely defected sperm compared to those with two copies of *Tnp2* and no *Tnp1* (producing 30% of the normal TP2 level). This indicates a unique role for TP2. It remains clear that the absence of both transition proteins leads to a persistence of endogenous DNA strand breaks in a fraction of ES suggesting that the combined DNA-condensing activity of these proteins is important to the genetic integrity of the developing male gamete. In this regard, it was demonstrated that TP1 facilitates DNA ligation and their detection in the nucleus coincide with a decrease in DNA fragmentation [Caron et al. 2001; Kierszenbaum 2001; Levesque et al. 1998]. In double TP knockout models, it is noteworthy that DNA fragmentation does not persist in some ES and that DNA repair, as seen by a decrease in TUNEL labeling, seems to proceed normally. Hence, the DNA condensing ability of the protamines (discussed below) may compensate for the lack of TPs.

Clearly, the potential link between the architectural DNA binding and condensing activity of these proteins and the stimulation of DNA repair needs to be further investigated.

PROTAMINES

Protamines are essential to DNA integrity and the ultimate compaction of the sperm head. The abnormal ratio of protamine 1 (PRM1) and 2 (PRM2) is found in some infertile men, suggesting that the relative levels of protamines are important for complete spermiogenesis. Protamine deficiency potentially has diverse origins. For a recent review, see [Carrell et al. 2007]. Mutations in the *PRM1* and *PRM2* genes have been identified in infertile men [Aoki et al. 2006b; Ravel et al. 2007]. Lower expression of one or both genes is associated with severe male infertility and also DNA damage in spermatozoa as evidenced by acridine orange staining [Aoki et al. 2006a; Cho et al. 2003]. Defects in the regulation of expression of protamines have been observed in infertile men as premature expression or retention of mRNA leads to infertility [Aoki et al. 2006b; Lee et al. 1995]. Also, proper post-translational modifications of protamines appear essential. Camk4, a multifunctional serine/threonine protein kinase may play a role in the exchange of TPs to protamine, as CAMK4 phosphorylates PRM2 *in vitro* and Camk4-null mice demonstrated prolonged retention of TP2 [Wu et al. 2000]. Moreover, the final step of this chromatin remodeling, the creation of inter- and intra-molecule disulfide bonds, is also known to affect fertility as suggested by the higher sensitivity of DNA damage of marsupial spermatozoa that do not possess such a capacity [Bennetts and Aitken 2005].

ENDOGENOUS DNA FRAGMENTATION AND REPAIR AS PART OF NORMAL SPERMIOGENESIS

Mammalian elongating spermatids (ES) have a unique nuclear status. McPherson and Longo [1992] first observed that steps 12 and 13 of spermiogenesis in the rat showed DNAse I hypersensitivity. Even without DNAse treatment, ES were labeled by nick translation, suggesting endogenous DNA fragmentation. They later postulated the implication of type II topoisomerases in the process of relaxing DNA supercoiling for the final protamination of sperm DNA [McPherson and Longo 1993]. Other reports suggested the involvement of topoisomerases during this process [Chen and Longo 1996; Cobb et al. 1997; Roca and Mezquita 1989]. In agreement with these observations, we demonstrated that 100% of spermatids undergo transient DNA breaks during steps 9 to 12 of mouse spermiogenesis as evidenced by TUNEL positivity [Marcon and Boissonneault 2004]. As demonstrated earlier by McPherson and Longo [1993] and confirmed later by our group, condensed spermatids (steps 14-16 in mouse) and epididymal spermatozoa, showed little to no DNA fragmentation after deprotamination and decondensation respectively, confirming the transient character of DNA fragmentation during spermiogenesis (Fig. 1).

In an effort to identify the enzyme responsible and the nature of these DNA breaks, we first used COMET assays to demonstrate that most of the DNA fragmentation encountered during the chromatin remodeling were double-stranded therefore supporting the involvement of a type II topoisomerase. In addition, we observed a severe decrease in DNA fragmentation when spermatids were incubated with two topoII inhibitors, namely etoposide and suramin [Laberge and Boissonneault 2005a]. Using confocal microscopy, we recently confirmed the presence of topoisomerase IIβ (TOP2B, topoIIβ) distributed in foci during steps 9 to 13 of mouse spermiogenesis but did not observe the presence of the α isoform at these steps [Leduc et al. unpublished results]. Interestingly, we identified the presence of tyrosyl-DNA phosphodiesterase 1(TDP1), an enzyme known to resolve topoisomerases-mediated DNA damage [Interthal et al. 2005; Nitiss et al. 2006; Raymond and Burgin 2006]. The presence of TDP1 is coincident with the appearance of topoIIβ in ES. Thus, one can hypothesize that TDP1 actively removes topoIIβ cleavable complexes during spermiogenesis, leaving a DSB. TDP1 was first identified by mass spectrometry following co-immunoprecipitation of hyperacetylated histone H4 (AcH4) from sonication resistant spermatid extracts (unpublished results) and later by immunofluorescence and immunoblots. The association of a repair enzyme such as TDP1 with AcH4 is consistent with the known requirement of histone hyperacetylation at sites of damage [Costelloe et al. 2006].

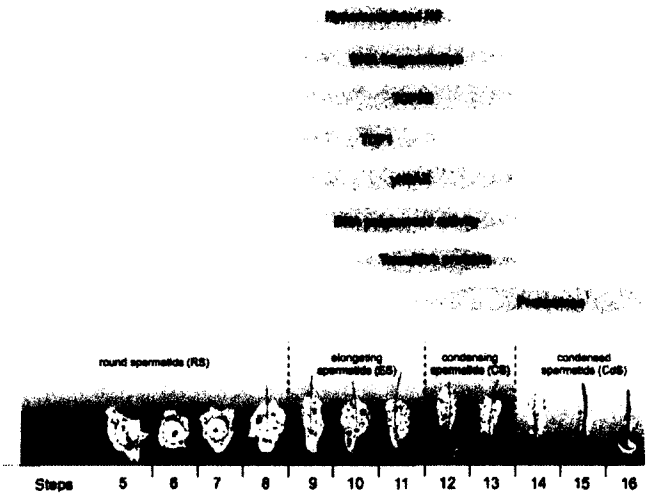

FIGURE 1 Schematic representation of immunofluorescence expression of different proteins or activity in the nucleus of spermatids during mouse spermiogenesis.

In this respect, Meyer-Ficca and colleagues [Meyer-Ficca et al. 2005] demonstrated the presence of poly(ADP)ribosyl polymers in rat ES as well as γH2AX foci. Consistent with these observations, the PARP2 knockout mouse was associated with severely compromised differentiation of spermatids and delays in elongation [Dantzer et al. 2006]. We confirmed the presence of γH2AX foci in mouse spermiogenesis and correlated this with TUNEL positivity, suggesting a genome-wide DNA damage response (DDR) as part of the normal program of spermiogenesis. Hence, DNA fragmentation shown by TUNEL is not an artefact generated by tissue fixation, as γH2AX is a biological marker of double-strand breaks (DSBs). Because of the presence of TDP1, it is tempting to speculate that part of the topoII-generated double-stranded breaks may not be processed correctly in the context of such an important chromatin remodeling and condensation. The activation of H2AX and the poly (ADP)rybosylation of histones at break sites therefore act as markers of the resulting damage and the DNA repair process may ensue independently from topoII since no fragmentation is observed in condensed spermatids and the presence of DNA polymerase activity at these late steps has been demonstrated [Hecht and Parvinen 1981]. DNA polymerase activity in non-replicating cells is indeed associated with repair. We have also found endogenous DNA polymerase activity present in elongating and condensing spermatids in accordance with those of others [Hecht and Parvinen 1981], demonstrating a unique DNA polymerase profile of ES. Interestingly, deletion of pol λ in mice, a member of the X family of DNA polymerase, leads to infertility. However, this is apparently due to a lack of motility rather than genomic instability, as microinjection of *pol* λ-null mice produces normal offspring [Kobayashi et al. 2002]. Consequently, considering DNA strand breakage and the signature of the repair activity, higher p53 levels found in ejaculates of infertile men [Sakkas et al. 2002] may not be related to apoptosis but rather to DNA repair.

FURTHER EVIDENCE OF A DNA REPAIR SYSTEM IN SPERMATIDS

Elongating spermatids, being haploid, cannot undergo homologous recombination (HR). Thus, we have hypothesized that the spermatid must rely on the more error-prone non-homologous end joining (NHEJ) for the repair of their endogenous DSBs [Leduc et al. unpublished results]. Components of NHEJ have been previously observed in spermatids [Goedecke et al. 1999], although not considered because of their lower level compared to other

germinal cell types. We detected the presence of phosphorylated DNApkcs at the condensing steps of spermiogenesis [Leduc et al. unpublished results]. DNApkcs is a member of the phospho-inositide 3-kinase family and usually part of the NHEJ. Evidence from the literature indicates that other DNA repair systems may play a role during spermiogenesis. Elements of the base excision repair (BER) have been identified in elongating and elongated spermatids [Olsen et al. 2001]. In addition, there is evidence that mismatch repair (MMR) involving the MSH2 protein, is playing a role during spermiogenesis. Using a Huntington disease mouse model, carrying a high number of CAG repeats, McMurray and Kovtun demonstrated that the repeat expansion was occurring between round spermatids and spermatozoa [McMurray and Kortun 2003]. Interestingly, the deletion of MSH2 in this mice model abolished the expansion [Kovtun and McMurray 2001], suggesting that MSH2 is active when extensive DNA repair occurs. Additional efforts are needed to identify the proteins involved in the repair process of ES.

CONCLUSION AND PERSPECTIVES

There is now an increasing interest in the impact of paternal age on fertility and embryo development. Recently, many reports suggested a clear relationship between paternal age and diverse pathologies in offspring such as autism, schizophrenia and Down's syndrome [Fisch et al. 2003; Malaspina et al. 2001; Reichenberg et al. 2006]. Others proposed a link between the age of the father and miscarriage [Kleinhaus et al. 2006]. Increased DNA fragmentation as determined by SCSA, mutagenesis and defective repair processes that occur with age have been proposed as underlying etiological factors [Marchetti and Wyrobek 2005; Wyrobek et al. 2006]. Interestingly, Schmid and colleagues [Schmid et al. 2006] explored sperm DNA damage and the influence of several lifestyle factors in healthly male non-smokers and found aging to be associated with single-strand breaks. Aside from the age-related effect, they also observed that caffeine consumption produces increased sperm DNA damage associated with DSBs. Quite interestingly, caffeine is a known inhibitor of members of the phospho-inositide 3-kinase family, implicated in many processes in meiotic cells. Recent published results indicate that caffeine may also lead to inactivation of H2AX and DNA repair (NHEJ) [Rybaczek et al. 2007]. Thus, impairment in signaling and DNA repair during spermiogenesis may likely result in persistent DSBs in mature spermatozoa.

This paper suggests that spermiogenesis has probably been overlooked as an important source of genomic instability. Spermiogenesis may, in fact, be the most crucial period of spermatogenesis because, despite their haploid character, spermatids must resolve these programmed double-stranded breaks. Given the limited repair capacity of the oocyte many idiopathic cases of infertility or embryo loss may be understood by a better knowledge of the chromatin steps of spermiogenesis. One must consider the distribution of strand breakage within the haploid genome rather that by the total number of break sites. Further investigation should provide important clues regarding the genetic consequences of the endogenous DNA strand breaks and repair in spermatids.

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Annexe 4 : Article soumis : Step-specific sorting of mouse spermatids by FACS, Simard, Leduc *et al.*, 2012

Step-specific sorting of mouse spermatids by FACS

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ABSTRACT

The differentiation of mouse spermatids is one critical process for the production of a functional male gamete with an intact genome to be transmitted to the next generation. So far, molecular studies of this morphological transition have been hampered by the lack of a method allowing adequate separation of these important steps of spermatid differentiation for subsequent proteomic and genomic studies. Earlier attempts at proper gating of these cells using flow cytometry may have been difficult because of a peculiar increase in DNA fluorescence in spermatids undergoing chromatin remodelling. Based on this observation, we provide details of a simple flow cytometry scheme, allowing reproducible purification of three populations of spermatids, each representing a different state in the nuclear remodelling process.

Key words: spermatids, DNA, flow cytometric cell sorting, spermatogenesis, spermiogenesis

INTRODUCTION

Haploid round spermatids differentiate into spermatozoa by a process called spermiogenesis. This involves many different steps including the acquisition of a flagellum, chromatin and cytoskeleton remodeling, condensation of the nucleus as well as the loss of most of the cytoplasm. These unique cellular events must be finely regulated in order to produce a mature functional gamete with an intact genome suitable for fertilisation. Spermiogenesis cannot be studied *in vitro* since no reliable cell culture system has so far been able to support progression through the different steps of the process. *In vivo*, proper transitions through the different steps of spermiogenesis are crucial for the natural functional integrity of the male gamete. Successful purification of spermatids according to their differentiation steps has never been accomplished with a level of enrichment sufficient to allow molecular characterization of spermiogenesis (25-29). For instance, purification of key steps of the spermatidal differentiation would be especially useful to study the developing acrosome, formation of the midpiece, cell junction dynamics, RNA dynamics or the chromatin remodeling process (25, 30-33). Purification of spermatids has been hampered by their progressive morphological transformation, the lack of known stage-specific external biomarkers, and their peculiar shape and size.

In order to better characterize the molecular events associated with the nuclear transition in spermatids, we sought a reliable method allowing the separation of spermatids into three morphological groups namely "round", "elongating" and

"elongated" with minimum contamination from other germ cell populations. Here, we describe a simple flow cytometry approach to separate mouse spermatids, as well as other cells of the seminiferous epithelium, with high purity (95-100%) based on their apparent DNA content.

General principles of spermatid sorting by flow cytometry

Although most male germ cells display a direct relationship between DNA staining and ploidy (DNA content), we noticed that this positive correlation is no longer valid in spermatids. This stems from our early observation that seminiferous tubule sections show variable intensity of DNA staining throughout the different spermiogenesis steps (34). We surmised that this was likely associated with the formation of their peculiar chromatin structure. DNA staining is indeed consistent with their haploid set of chromosomes from steps 1 to 7 (round spermatids). However, a striking increase in fluorescence intensity is observed around the onset of nuclear reorganization and chromatin remodeling (step 8) reaching a peak value around the onset of nuclear condensation (steps 11-12). Following condensation of the nucleus, DNA staining intensity decreases until spermiation (step 16).

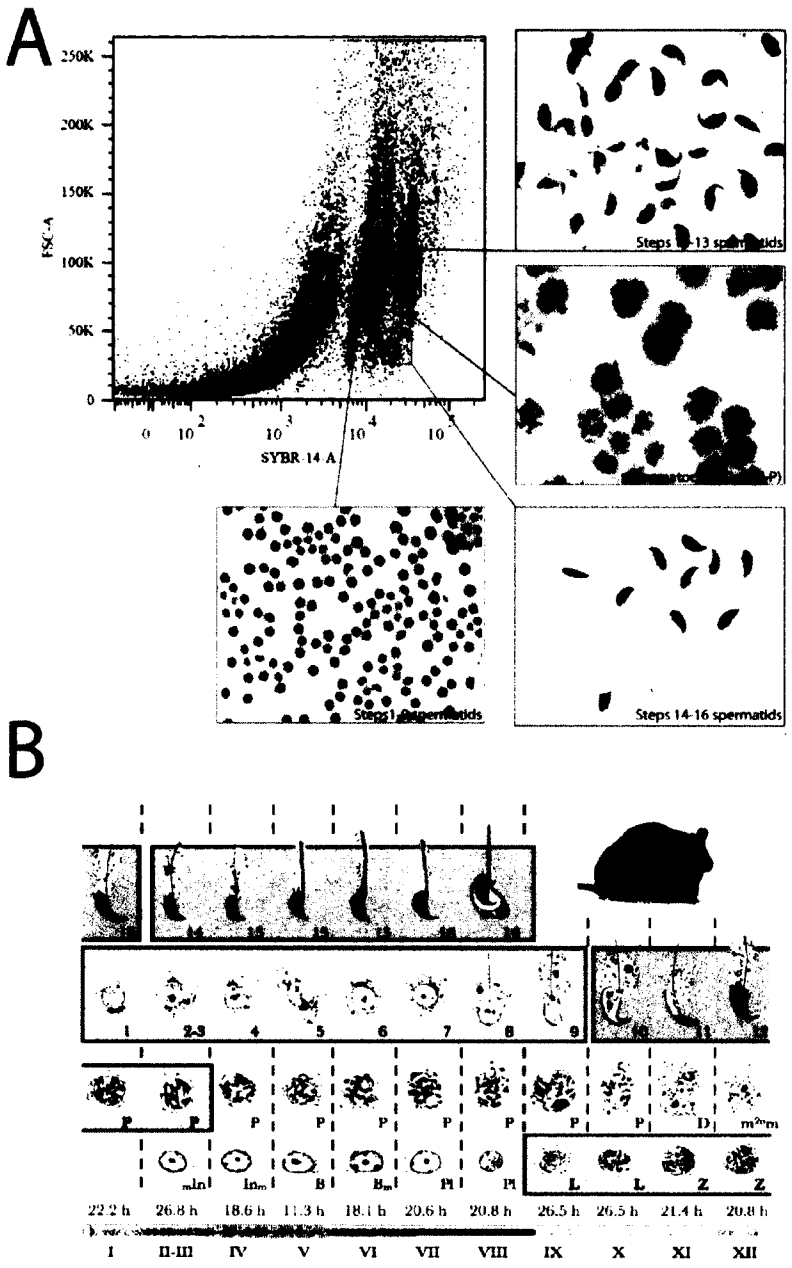

Figure 1. Sorted germ cell populations by flow cytometry
(A) Upper left panel, Dot plot of size (FSC-A) vs. DNA staining (SYBR-14) of four sorted germ cell populations. Bottom and right panels, DAPI-stained images of the sorted cells visualized by epifluorescence microscopy. **(B)** Mouse spermatogenesis stage map showing the sorted cell populations and correspondance to the transillumination patterns of seminiferous tubules, adapted from Russel *et al.* (1990) (1) and Kotaja *et al.* (2004) (2).

Using a permeable DNA dye (SYBR-14) normally used to label sperm cells (35), we integrated the variation in DNA labeling intensity observed in differentiating spermatids into our flow cytometry gating strategy. As we were looking for elongating spermatids (steps 9-12) in the tetraploid range of DNA intensity, we also observed that elongating spermatids were very close to spermatocytes in apparent size (see Figure 1A). However, by applying gates combining granulosity, size and DNA intensity, we were then able to separate elongating spermatids (steps 10-13) from spermatocytes (Figure 2). This strategy allowed to sort four distinct cell populations of interest with purity ranging from 95 to 100%, namely spermatocytes (mainly leptotenes, zygotenes and early pachytenes), round spermatids including early elongating spermatids (steps 1-9), elongating spermatids (steps 10-13) and elongated spermatids (steps 14-16) (Figure 1B). Details of the gating strategy are provided in Figure 2.

Outline of the protocol

Briefly, testes are excised from adult male mice euthanatized under CO_2 and homogenized with scissors in the sorting buffer. Germ cells are then fixed with ice-cold ethanol and DNA stained using SYBR-14. Primary spermatocytes, round and early elongating spermatids (steps 1-9), elongating spermatids (steps 10-13) and elongated spermatids (steps 14-16) are sorted according to their apparent DNA content, size and granulosity using a 488nm laser-equipped cell sorter.

MATERIALS

REAGENTS

- Male mouse over 42 days of age
- Distilled water
- Isoflurane (ABBOT N°B506 DIN 02032384)
- Fetal bovine serum or FBS (Wisent, 090150)
- NaCl (Fisher, S271-3)
- KCl (Fisher, P217)
- Na_2HPO_4 (Fisher, S369)
- KH_2PO_4 (BioShop, No.:9E11661)
- EDTA (Bioshop, EDT001.500)
- HEPES (Sigma, H3375-500G)
- 100% ethanol (Les alcools de commerce, 092-09-11N)
- SYBR-14 (Invitrogen, LIVE/DEAD Sperm Viability Kit, 7011)

EQUIPMENT

- 1.5mL eppendorf tubes (PROGENE #24-MCT-150-C)
- 5mL polypropylene round bottom tubes (BD Falcon, cat # 352063)
- 15mL polypropylene conical bottom tubes (BD Falcon, cat # 352097)
- 50mL polypropylene conical bottom tubes (BD Falcon, cat # 352070)
- Dissection kit
- TEC4 anaesthetic vaporizer (Ohmeda No.1160526)
- CO_2 gas tank (PRAXAIR C799117902)
- O_2 gas tank (PRAXAIR O254130501)
- Homemade mouse gas chamber
- 100-1000 µL tips (Corning Incorporated, cat # 4846)
- 40µm cell strainer (BD Falcon, REF: 352340)
- 50-micron sample line filters (BD biosciences, cat.649049)
- Vortex mixer (Labnet international, inc. S0200)
- Dynac centrifuge (Clay Adams, cat.no. 0101)
- Celltrics 50µm filters (Partec, cat. 04-004-2327)
- 488nm laser-equipped cell sorter (here, FACS ARIA III, BD)
- Mini Labroller rotator (Labnet International Inc., Edison, NJ)
- 4°C refrigerator
- ice

REAGENT SETUP

- 1X PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4. Autoclaved and stored at RT.

- Sorting Buffer: 1X PBS, 1mM EDTA pH8, 25mM HEPES pH7 and 1% heat-inactivated fetal bovine serum. Fresh solution, 0.2µm filtered and kept at 4°C.

PROCEDURE

Part 1: Tube preparation (30 minutes hands-on, overnight incubation)

1. The day before cell sorting, add 1-2mL of heat-inactivated FBS in 5mL polypropylene round bottom tubes, and in 15mL and 50mL polypropylene conical tubes.

*Critical step: Every tube used in the protocol must be coated, FBS coating prevents germ cells from sticking to tube walls.

2. Slowly mix vertically to coat the tubes uniformly on a rotator at 4°C overnight.

3. On the next day, remove FBS from coated tubes.

*Critical step: The 5mL conical propylene tubes and the 15 and 50mL tubes used for cells preparation (steps 3-16) must be completely emptied of FBS to prevent fanning (i.e. imprecise deviation of the side streams) which may lead to a loss of sorted cells or contamination of other sorting tubes during cell sorting caused by a high concentration of FBS. A small residual volume of FBS (about 100-200µL) should be left in the 5mL conical tubes used for collecting purified cells (Step 17).

Part 2: Cells preparation (100 minutes)

4. On the next day, anaesthetize one male mouse with O_2/isoflurane (induction at 5% then maintained at 2%) and sacrifice by CO_2 asphyxiation.

5. Excise and decapsulate 2 testes, and mince with small scissors in 1mL of sorting buffer in a 1.5mL tube.

6. Flush germ cells from seminiferous tubules by gentle up and down pipetting in the 1.5mL tube, first with a truncated 100-1000µL tip, and then with an intact 100-1000µL tip.

7. Remove debris and clumps by one filtration step using a 40µm cell strainer and collect filtrate into a 50mL conical tube (previously coated in step 1).

8. Wash the filter once with 500µL of sorting buffer and transfer filtrate into a 15 mL conical tube (previously coated in step 1).

9. Adjust total volume to 3mL with sorting buffer.

10. Add 16µL of 50mM EDTA pH 8 per milliliter of cell suspension (48µL for 3mL) to obtain a final concentration of 0.8 mM EDTA.

11. To fix germ cells, slowly add 3 volumes of ice-cold 100% ethanol with gentle mixing (vortex on a low setting) which will produce a milky suspension.

*Critical step: Slowly adding ethanol is crucial and important to preserve the integrity of the cell preparation. We noted that a rapid addition of ethanol causes a high percentage of cells lysis resulting in a major decrease of sorting efficiency.

12. Incubate cells on ice for 15 minutes with occasional mixing by inversion.

13. Centrifuge cells suspension at 800*g* for 5 minutes and remove the clear supernatant.

14. Gently resuspend the fixed germ cells in 2mL of sorting buffer.

15. Add 4 μL of SYBR-14 DNA dye and incubate for 30 minutes (protected from light).

Part 3: Cell sorting (3 to 8 hours)

16. Set up the cell sorter following the Supplementary Procedures added to this protocol.

17. Immediately before sorting, filter cells using Celltrics 50μm filters in a 5mL polypropylene round bottom tube (previously coated in step 1)

18. Wash the filter extensively with 1-2mL of sorting buffer.

19. Sort fixed germ cells with a 488nm laser-equipped cell sorter (here, a BD FACS ARIA III flow cytometer) following the gating scheme detailed in Figure 2. Collect purified fractions in 5mL polypropylene round bottom tubes (previously coated in step 1) on ice.

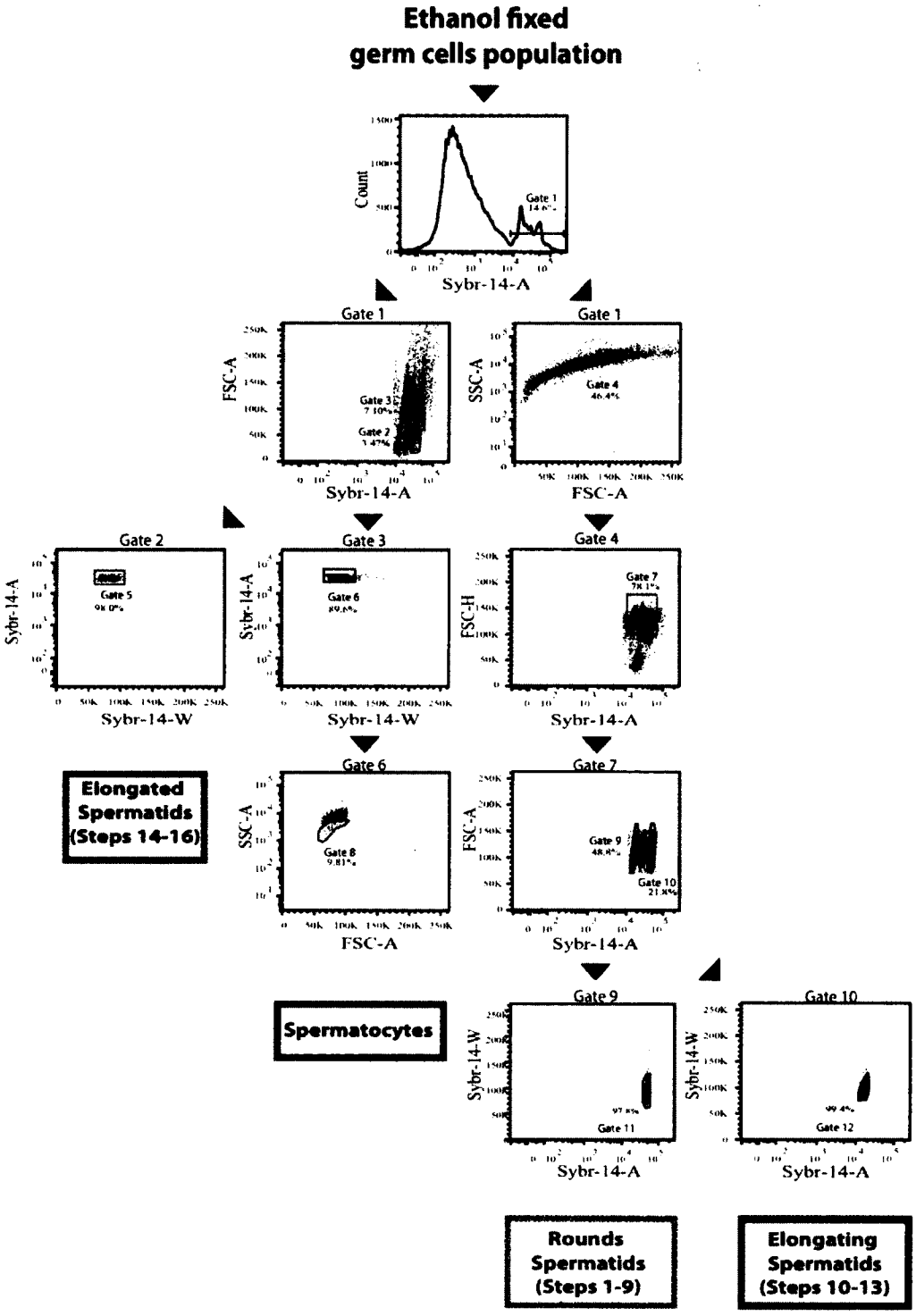

Figure 2. Detailed gating strategy to sort ultrapure spermatids populations
Schematic representation of the gating strategy used to sort spermatocytes, steps 1-9 spermatids, steps 10-13 spermatids and steps 14-16 spermatids simultaneously with a 488 nm laser-equipped cell sorter. Percentages represent selected cells compared to previous gate.

TROUBLESHOOTING

TABLE I: Troubleshooting cell sorting problems

Step	Problem	Possible reason	Possible solutions
18	Unable to sort because of a fanning issue	FBS concentration too high	Remove as much FBS as possible from tubes.
18	Number of cells analyzed decreases over time	Clumped cells	Extra filtration step Constant vortexing during sorting (300 rpm) Change filter on the sample line in the cell sorter
18	Too many events	Cell concentration is too high	Dilute cell suspension with sorting buffer
18	Too much debris	Cell integrity affected during cell preparation	Add ethanol slowly (step 11) Resuspend cells gently (step 14)
18	Unstable DNA staining of cells	SYBR-14 concentration is too low	Increase SYBR-14 concentration to saturate DNA content of cells
18	Low purity of sorted cells	Sorting gates too large Number of event per second is too high Flow rate is too fast	Use narrower gates to sort cell populations Decrease event/s to a maximum of 5000 events/s Set the Flow rate to 3.0 maximum

TIMING

Day 1

Step1, Preparing FBS-coated tubes: 15 min

Step 2, Overnight incubation: about 16 h

Day 2

Step 3, Removing FBS from tubes: 15 min

Steps 4-9, Cell preparation: 40 min

Steps 10-14, Cell fixation: 30 min

Steps 15, DNA staining: 30 min

Steps 16-18, Cell sorting: 3-8 h

ANTICIPATED RESULTS

TABLE 2: Anticipated results

Step	Anticipated results
11	After the addition of ice-cold ethanol, the cell suspension will have a milky and clumpy appearance.
13	The white cell pellet should have a volume of 1-2mL and should be easily resuspended with gentle "up and down" pipetting.
18	Expect about 60 to 80% of events that are SYBR-14 negative (cell debris) and about 20 to 40% of cells (SYBR-14 positive).

TABLE 3: Sorted germ cell populations from total germinal cell suspension by flow cytometry

	Majority cell type observed	**Percentage of purity**	**Number of cells (8h sorting)**	**Contaminants**
Population 1	Steps 1-9 spermatids	99-100%	6,000,000	Step 10 spermatids
Population 2	Steps 10-13 spermatids	95-98%	2,000,000	Round spermatids Secondary and pachytene spermatocytes
Population 3	Steps 14-16 spermatids	99-100%	1,000,000	Round spermatids
Population 4	Leptotene, zygotene and early pachytene spermatocytes	100%	500, 000	-

BOX 1: Transillumination pre-selection (timing 2-3h)

To further define the cell population to be sorted by flow cytometry, it is possible to pre-select staged seminiferous tubule sections (about 2 to 10 mm in length) using the transillumination pattern as described previously (see Figure 1B). Using the strategy described by Kotaja *et al.*(2), we were able to select specific stages by dissecting tubules showing pale/weak spot and dark patterns corresponding to stages VIII to I-II and stages VI to VIII respectively (see Figure 1B).

Step PS1. Instead of mincing the testes with scissors, use small tweezers and microscissors to disperse the seminiferous tubules in a Petri dish containing several mL of cold sorting buffer. Use a transillumination binocular (at 16-40X magnification).

Step PS2. The regions of interest are resected as defined by Kotaja *et al.* (pale, weak spot, strong spot, dark), and tweezers are used to transfer selected tubules to a second Petri dish (kept on ice) in a 200µL drop of sorting buffer.

Step PS3. After 1-2h of tubule selection, extrude the cells from the tubules using one pair of tweezers to maintain the tubule at one end and squeezing the tubules with another pair of tweezers starting close to the first one and pushing the cells gently toward the other end. After each extrusion, remove the emptied tubule that sticks to the tweezers using a precision wiper tissue.

Step PS4. After extrusion of all tubules, perform several up and down aspirations with a 20-200µL micropipette so as to obtain a homogenous cell suspension.

Step PS5. Continue the protocol from step 10.

TABLE 4: Sorted germ cell populations using transillumination pre-selection

"Pale/weak spot" tubules pre-selection (stages VIII to I-II)	**"Dark" tubules pre-selection (stages VI to VIII)**
Steps 1-2 and 9 spermatids (population 1)	Steps 6 to 8spermatids (population 1)
Steps 10-13 spermatids (population 2)	Steps 15-16 spermatids (population 3)
Leptotene, zygotene and early pachytene spermatocytes (population 4)	Pachytene spermatocytes (population 4)

ALTERNATIVE METHODS

Approaches using gravity purification such as percoll gradient (36) or bovine serum albumin sedimentation(25), provide a good yield of intact germinal cells but lack definition between some cell types such as meiotic tetraploid cells and spermatids (25, 29). Moreover, these techniques require special devices (often homemade) that may not be readily available for many laboratories; it is therefore much more difficult

to reproduce the conditions and procedures described in the literature without cumbersome trial and error. These methods are also time-consuming and very sensitive to vibrations (the apparatus is usually kept at 4°C to maintain cell viability) but, when successful, provide several million purified live cells per population. However, the purity of these populations is the main weakness of these techniques as it rarely reaches more than 80-90%; in certain cases, a contamination of 10-20% of other cell types when measured by DNA, RNA or protein content may very well represent a critical bias.

When seeking to improve purity of cell populations some have combined gravity sedimentation to other methods. One of the most effective approach is to use immature mice to limit the number of different cell populations representing later stages (25); one can take advantage of the first wave of spermatogenesis and obtain a cell preparation containing germinal cells up to a given differentiation stage based on their sequential appearance after birth. However, this combined approach requires more animals to compensate for the limited amount of starting material and yet does not resolve the lack of definition of the sedimentation methods. In addition, such an approach cannot be practically applied for later cells types such as spermatids, but mainly for spermatogonia and primary spermatocytes (25). Moreover, there is some evidence that the first wave of spermatogenesis may harbour cells with slightly different properties than that of the cycling epithelium of mature mice (37).

It is also possible to sort spermatidal nuclei using the metachromatic dye acridine orange based on the chromatin condensation state (13) but, because of its lack of specificity between single-strand DNA and RNA, we opted for a DNA double-strand-specific dye. Vitamin A synchronization of spermatogenesis was also used to improve germinal cell purification as it narrows down the number of stages present in the testes of a treated animal. However, this procedure was mainly used to synchronize spermatogenesis in rats, with some reported success in mice (27, 38, 39). From our own experience, vitamin A synchronization in mice is time-consuming, rarely gives reproducible results and produces harmful side effects raising concern about the cellular integrity of the seminiferous epithelium.

LIMITATIONS

The main constraint of the method described in this paper may be the limited yield of sorted cells. The separation power of flow cytometry provides three highly purified haploid cells populations representing important differentiation steps amenable to molecular studies of the process. The high level of purity is somewhat obtained at the expense of the number of sorted cells. However, a single day of sorting under the conditions described here would yield a minimum of 500 000 spermatocytes, and up to millions of cells of other populations, which is sufficient for immunoblot analyses of much less abundant proteins (see Figure 3) or DNA extraction. Alternatively, gates can be expanded to increase the number of sorted cells, eventually resulting in a small decrease in population purity, which can be acceptable for some studies. Furthermore, ethanol-fixed germ cells stained with SYBR-14 are very stable at

4°C and can be sorted for more than 8 hours with limited supervision and adjustments. Ultimately, several days of sorting can be pooled to obtain a sufficient numbers of cells for any application. To enrich the starting material for given stages of the seminiferous epithelium, we suggest the transillumination pre-selection step described in BOX 1.

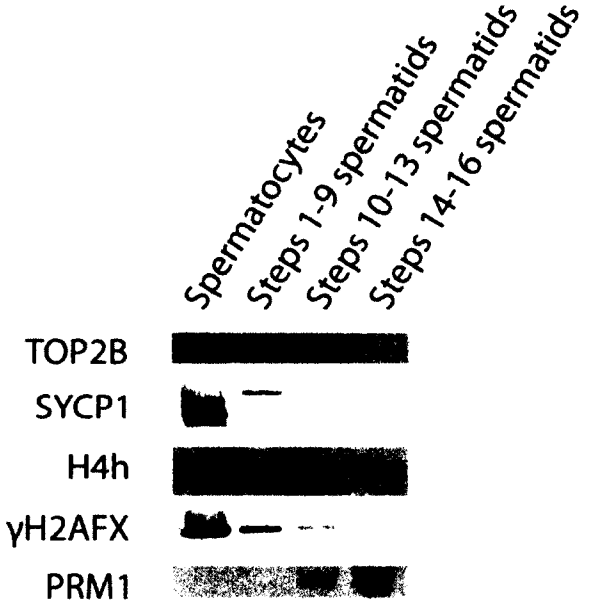

Figure 3. Detection of stage-specific marker proteins in sorted germ cell populations

Immunoblot analysis of topoisomerase II β (TOP2B), Synaptonemal complex protein 1 (SYCP1), hyperacetylated Histone H4 (H4h), phosphorylated histone variant H2AFX (γH2AFX) and protamine 1 (PRM1) in spermatocytes, steps 1-9 spermatids, steps 10-13 spermatids and steps 14-16 spermatids sorted by flow cytometry. For abundant proteins such as SYCP1, H4h and γH2AFX, standard 15% SDS-PAGE immunoblots were performed with 180 000 cells for each population, whereas 525 000 cells were used for the detection of TOP2B. For PRM1 analysis, basic proteins from 525 000 cells per population were acid extracted, separated in a 15% acid-urea polyacrylamine gel and detected by immunoblot, as described by Torregrosa *et al.* (40). Estimation of the cells was based on the number of events sorted by flow cytometry for each population.

APPLICATIONS

Sorted spermatids can be used for a variety of experiments. DNA from these cells can easily be extracted using commercial genomic DNA purification kits (Qiagen, QIAmp DNA Mini Kit cat# 51304) and demonstrated to be of proper quality for PCR analyses and deep sequencing (data not shown). We also showed that sorted spermatids could be used for protein analyses (Figure 3). The presence of known keys nuclear proteins, including histone variants and histone post-translational modifications (5, 34, 40-42), was demonstrated using a standard SDS-PAGE immunoblot protocol for the detection of TOP2B, SYCP1, hyperacetylated histone H4 (H4h) and γH2AFX, whereas the presence of protamine 1 (PRM1) was detected using an acid-urea denaturing immunoblot. These stage-specific markers also confirmed the high purity of the sorted spermatids and spermatocytes. Hence, highly pure germ cell populations sorted using this approach are suitable for proteomic and genomic analyses, as well as other applications.

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Contributions

F.L. M.A. and G.A. contributed to the data (early version of the protocol and exploration data). O.S., F.L. and M.A. did the experiments and analyzed data. O.S., M.G. and M.B. did additional experiments (applications). I.M. and M.R. provided FACS expertise and analysis and wrote the sorting protocol. O.S., F.L. and G.B. planned experiments and wrote the paper.

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Competing financial interests

The authors declare no competing financial interests.

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SUPPLEMENTARY PROCEDURES

Cell sorter set up and sorting

We used a 4-Laser (405nm - violet, 488nm - blue, 561nm - yellow-green, 633nm - red) 20-parameter BD FACSAria III. The BD FACSDiva 6.1.3 software was used to visualize and analyze the data.

A. Sterilize the BD FacsAria III by running 70% (vol/vol) ethanol as sheath fluid during the fluidics shutdown procedure ("Cytometer" menu in the FACSDiva software) prior to sorting.

B. Prepare and filter PBS 1X with a 0.22 µm filter to avoid the presence of any crystals and contaminant. Fill the sheath tank to the upper weld line on the inside of the tank.

C. Start the BD FACSDiva software and the BD FACSAria III to warm up the lasers for at least 30 minutes prior sorting. Run two fluidic startup processes ("Cytometer" menu of the FACSDiva software) to flush out the ethanol from the fluidics. This restores fluidics with normal sheath fluid (PBS).

D. Clean the sort block and the deflection plates with distilled water and dry them thoroughly. Sonicate a 100-μm nozzle placed in a tube of distilled water for 2 min in a sonication bath. Wipe the nozzle thoroughly.

E. Insert the 100-μm nozzle into the flow cell and turn the nozzle-locking lever clockwise to the 12:00 position. Set the sheath pressure to 20 psi. We have also tested a 130-μm nozzle at 10 psi and found no obvious difference. *Critical step: Make sure that the stream is straight and hits the center of the waste aspirator by changing the angle of the sort block.

F. Turn on the stream and adjust the amplitude to obtain target values for frequency (around 30), Drop 1 (around 150) and Gap (around 12) and to establish a stable droplet breakoff pattern (few satellite drops). Turn off attenuation. Laser delay was set to 0 for the blue, -77.39 for the red, 37.33 for the violet and -39.85 for the yellow-green lasers. Areas scaling was set to 1.14 for the blue, 1.0 for the red, 0.75 for the violet and 0.96 for the yellow-green lasers. *Critical step: Make sure, using a Test Sort (in the "Side Stream" window of the FACSDiva software), that side streams are not fanning and adjust the voltage sliders of each side stream so that they hit the middle of test collection tube. If the center stream is not tight, adjust the 2nd, 3rd, and 4th Drop settings to constrain the center stream.

G. Start the Cytometer Setup and Tracking application in the FACSDiva software ("Cytometer" menu). Use the BD Cytometer Setup and Tracking Beads (BD biosciences, cat. 642412) at 1 drop per 350μL to automate the characterization and tracking of the cytometer's performance. Quit the Cytometer Setup and Tracking application. Apply CS&T setting, make sure the stream parameters still allow a stable droplet breakoff pattern, and turn on the Sweet Spot (in the "Breakoff window"). Optimize the Drop Delay value using BD Accudrop Fluorescent Beads (cat. # 345249, BD Biosciences). *Critical step: This step is critical to make sure that the cells of interest sort into a side stream. The beads and the optical filter are used to achieve close to a 100% droplet deviation. Adjust the micrometer dial to obtain brightest bead spot at the center. Set the sort precision to "Initial" and finally to "Fine tune" in the "Sort layout" window.

H. Place a FSC 1.5 ND (neutral density) filter at the left end of the FSC detector block. Set the window extension to 2.00μs (in the "Laser" tab in the "Cytometer" window).

I. The FSC Area scaling was set to 1.00.

J. *Critical step: Before loading sample tube, place a sample filter line of 50μm at the end of the sample line to avoid clumping. To do so, select "Change Sample Filter" in the "Cytometer" menu.

K. *Critical step: Test the composition of the sample fluid to achieve good separation without fanning between side stream and to prevent the cells from sticking to the BD polypropylene tubes.

L. Load the sample tube into the FACS instrument. Select a sample agitation of 300 rpm and a sample temperature of 4°C. Select "Acquire Data" in the "Acquisition Dashboard".

M. If necessary, dilute the sample to achieve an appropriate event rate of a maximum of 5000 events/s (at a maximum flow rate of 3.0). *Critical step: These limits must be respected strictly to maintain the purity of the sorted cells.

N. To analyze and sort the cells, optimize the FSC threshold value to eliminate debris without interfering with the population of interest. In logarithmic scale, adjust the voltage of the PMT detector with the 530/30 filter in order to make sure that the fluorescence signal of the total population fits within the scale, and gate on the positive population (P1). *Critical step: Make sure that the concentration of SYBR-14 is saturating to avoid fluorescence fluctuations during the sorting period.

O. Produce a Forward Scatter-area/Side Scatter-area plot of the SYBR-14 positive population and adjust the Forward Scatter-area (FSC-A) to around 110V in linear scale, and the Side Scatter-area (SSC-A) to around 150V in logarithmic scale to visualize all the events while excluding very large cells and cell aggregates.

P. Set gates according to the Figure 3.

Q. In the "Sort Layout" window, set the "Sort Precision Mode" to "4-way purity" (Yield mask: 0, Purity Mask: 32, Phase Mask: 0). Select "Continuous" from the "Target Events" menu for continuous sorting. Add populations to sort in the field corresponding to the tubes (in the "Sort Layout" window). Populations with higher frequencies were placed in the middle whereas those with lower frequencies were

placed at the ends. Set the voltage plates to 2500 volts. During sorting, keep the sample collection tubes on ice.

Protein analysis by immunoblot of sorted cells

For abundant proteins, 180 000 cells from each population were loaded on a 15% SDS-PAGE gel and analyzed by immunoblot using the following primary antibodies: rabbit anti-H4h (cat. # 06-946, Millipore, Billerica, MA, USA), rabbit anti-SYCP1 (kindly provided by Paula E Cohen, Cornell University, Ithaca, NY, USA) and mouse anti-γH2AFX (cat. # ab22551, Abcam, Cambridge, MA, USA). For rare proteins, 525 000 cells from each populations were loaded on a 15% SDS-PAGE gel and analyzed by immunoblot using the rabbit anti-TOP2B primary antibody (cat. # sc-13059, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For PRM1 analysis, basic proteins from 525 000 cells from each population were acid extracted, separated on a 15% acid-urea polyacrylamide gel and detected by immunoblot, as described by Torregrosa *et al.* (40) using a mouse anti-PRM1 (cat. # Mab-001 Hup 1N, Briar Patch Biosciences LLC, Livermore, CA, USA). Goat anti-mouse IgG Alexa 680 (cat. # A21057, Invitrogen, Carlsbad, CA, USA) or goat anti-rabbit IgG IRDye 800CW (cat. # 926-32211, Li-Cor Biosciences, Lincoln, NE, USA) were used as secondary antibodies for immunoblots and membranes were revealed using an Odyssey imaging system (model: 9120, Li-Cor Biosciences). Quantification of cells for each lane was based on the approximated number of events of each population sorted by flow cytometry.

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