KNOBIL AND NEILL'S PHYSIOLOGY OF REPRODUCTION

FOURTH EDITION

VOLUME 1

Editors-in-Chief

TONY M. PLANT AND ANTHONY J. ZELEZNIK
University of Pittsburgh, Pittsburgh, PA, USA

Associate Editors

DAVID F. ALBERTINI
Kansas University Medical Center, Kansas City, KS, USA

ROBERT L. GOODMAN
West Virginia University, Morgantown, WV, USA

ALLAN E. HERBISON
Otago School of Medical Sciences, Dunedin, New Zealand

MARGARET M. MCCARTHY
University of Maryland School of Medicine, Baltimore, MD, USA

LOUIS J. MUGLIA
Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

JOANNE S. RICHARDS
Baylor College of Medicine, Houston, TX, USA

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Associate Editors

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Kansas University Medical Center, Kansas City, KS, USA

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Pair bonded prairie vole family from Chapter 48 (J. Balthazart and L. Young). Photo by Todd H. Ahern

Relationship between gonadotropin releasing hormone GnRH (red) and kisspeptin neurons (green) in a coronal section of the monkey hypothalamus from Chapter 28 (A.J. Zeleznik and T.M. Plant).

Mouse uterus showing vascular permeability at the sites of implantation detected after an intravenous injection of a blue dye on day 8 of pregnancy. From Chapter 38 (S.K. Dey). Per Dey, acknowledgement/credit not needed.

Top back. Scanning electron micrograph of a bull sperm interacting with ciliated epithelium in the sperm storage reservoir of the oviduct (Chapter 5, Lefebvre et al., 1995).

Middle back from David Albertini. Confocal micrograph of a horse oocyte based on collaboration between Elaine Carnevale, Colorado State University and David Albertini and John Bromfield of the Kansas University Medical Center.

Lower back from Chapter 28 (A.J. Zeleznik and T.M. Plant). Pulsatile LH release from the pituitary and multi unit electrical activity in the mediobasal hypothalamus recorded from an intact (left panel) and ovariectomized (right panel) rhesus monkey.

Contributors

Kjersti M. Aagaard Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine, Baylor College of Medicine, Houston, TX, USA

David F. Albertini Department of Molecular and Integrative Physiology, Institute of Reproductive Health and Regenerative Medicine, Kansas University Medical Center, Kansas City, KS, USA

Eugene D. Albrecht Departments of Obstetrics, Gynecology, and Reproductive Sciences and Physiology, University of Maryland School of Medicine, Baltimore, MD, USA

Steven M. Anderson University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA

William E. Armstrong Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN, USA

Richard J. Auchus Division of Metabolism, Endocrinology, and Diabetes, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA

Susan P. Bagby Department of Medicine, Division of Nephrology and Hypertension, Center for Developmental Health, Knight Cardiovascular Institute, Oregon Health and Science University, Portland, OR, USA

Jacques Balthazart GIGA Neurosciences, University of Liège, Liège, Belgium

April K. Binder Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

Jeffrey D. Blaustein Center for Neuroendocrine Studies, Neuroscience and Behavior Program, University of Massachusetts, Amherst, MA, USA

S. Marc Breedlove Neuroscience Program, Michigan State University, East Lansing, MI, USA

Paula J. Brunton Division of Neurobiology, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK

Jeeyeon Cha Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Shawn L. Chavez Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA, USA, Department of Obstetrics and Gynecology, Stanford University, Stanford, CA, USA, Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA

Paula E. Cohen Department of Biomedical Sciences, Cornell University, Ithaca, NY, USA

Gerard S. Conway Department of Endocrinology, University College Hospitals, London, UK

John F. Couse Taconic Farms, Albany Operations, Rensselaer, NY, USA

Geert J. de Vries Neuroscience Institute, Georgia State University, Atlanta, GA, USA

Emily DeFranco Department of Obstetrics and Gynecology, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Francesco J. DeMayo Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Sudhansu K. Dey Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Juan M. Dominguez Department of Psychology, University of Texas, Austin, TX, USA

Edward M. Eddy Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC, USA

Rafael A. Fissore Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA, USA

Loretta M. Flanagan-Cato Laboratory of Neuroendocrinology, Department of Psychology, University of Pennsylvania, Philadelphia, PA, USA

Harvey M. Florman Department of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA, USA

Nancy G. Forger Neuroscience Institute, Georgia State University, Atlanta, GA, USA

Douglas L. Foster Departments of Obstetrics, Gynecology, and Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, USA

Stephen Franks Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Hospital, London, UK

Jennifer R. Gardiner Division of Cancer Biology, Institute of Cancer Research, London, UK

Kathrin Gassei Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

George D. Giraud Department of Medicine, Center for Developmental Health, Knight Cardiovascular Institute, Oregon Health and Science University, Portland VA Medical Center, Portland, OR, USA

Robert L. Goodman Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV, USA

Andrea C. Gore Division of Pharmacology and Toxicology, The University of Texas at Austin, Austin, TX, USA

David R. Grattan Centre for Neuroendocrinology, University of Otago, Dunedin, New Zealand

Janet E. Hall Department of Medicine, Reproductive Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

Kate Hardy Institute of Reproductive and Developmental Biology, Imperial College London, Hammersmith Hospital, London, UK

Frances J. Hayes Department of Medicine, Reproductive Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

David Hazlerigg Department of Arctic and Marine Biology, University of Tromsø, Tromsø, Norway

Mark P. Hedger Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia

Jon D. Hennebold Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA

Allan E. Herbison Center for Neuroendocrinology and Department of Physiology, Otago School of Medical Sciences, Dunedin, New Zealand

Sylvia C. Hewitt Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

Stanley M. Hileman Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV, USA

Barry T. Hinton Department of Cell Biology, University of Virginia Health System, Charlottesville, VA, USA

J. Kim Holloway Department of Biomedical Sciences, Cornell University, Ithaca, NY, USA

Elaine M. Hull Department of Psychology, Florida State University, Tallahassee, FL, USA

Joan S. Hunt Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA

Mary Hunzicker-Dunn School of Molecular Biosciences, Washington State University, Pullman, WA, USA

E. Keith Inskeep Division of Animal and Nutritional Sciences, West Virginia University, Morgantown, WV, USA

Thomas Jansson Department of Obstetrics and Gynecology, Center for Pregnancy and Newborn Research, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

Sherri L. Jones Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, Montréal, QC, Canada

Kenneth S. Korach Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

Michael J. Large Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Jon E. Levine Department of Neuroscience, School of Medicine and Public Health, Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI, USA

Xiao-Feng Li Academic Department of Anatomy and Human Sciences/Division of Women's Health, School of Medicine, King's College London, Guy's Campus, London, UK

Hyunjung (Jade) Lim Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; Department of Biomedical Science and Technology, Konkuk University, Seoul, South Korea

Mark Lindgren Department of Urology, University of Illinois at Chicago, Chicago, IL, USA

Zhilin Liu Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Joseph S. Lonstein Neuroscience Program and Department of Psychology, Michigan State University, East Lansing, MI, USA

Paul S. MacLean University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA

Catherine A. Marler Neuroscience Training Program and Department of Psychology, University of Wisconsin, Madison, WI, USA

Kelly Mayo Department of Molecular Biosciences, Center for Reproductive Science, Northwestern University, Evanston, IL, USA

Erik C. Mazur Department of Obstetrics and Gynecology, Baylor College of Medicine, Texas Children's Hospital Pavilion for Women, Houston, TX, USA

Craig A. McArdle School of Clinical Sciences, Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, UK

Margaret M. McCarthy Departments of Pharmacology, Physiology, and Psychiatry, Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD, USA

Neil J. McKenna Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

James L. McManaman University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA

Sam Mesiano Department of Reproductive Biology, Case Western Reserve University, Cleveland, OH, USA, Department of Obstetrics and Gynecology, University Hospitals of Cleveland, Cleveland, OH, USA

Joan I. Morrell Center for Molecular and Behavioral Neuroscience, Rutgers University, Newark, NJ, USA

Louis J. Muglia Center for Prevention of Preterm Birth, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Makoto C. Nagano Department of Obstetrics and Gynecology, Royal Victoria Hospital, McGill University, Montreal, QC, Canada

Margaret C. Neville University of Colorado Denver, Anschutz Medical Campus, Aurora, CO, USA

Kevin T. O'Byrne Academic Department of Anatomy and Human Sciences/Division of Women's Health, School of Medicine, King's College London, Guy's Campus, London, UK

Peter O'Shaughnessy Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

Kyle E. Orwig Departments of Obstetrics, Gynecology and Reproductive Sciences, and Molecular Genetics and Developmental Biology, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Stephanie A. Pangas Department of Pathology and Immunology, and Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Gerald J. Pepe Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA, USA

Mariana Pereira Center for Molecular and Behavioral Neuroscience, Rutgers University, Newark, NJ, USA

Margaret G. Petroff Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA

James G. Pfaus Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, Montréal, QC, Canada

Bart T. Phillips Departments of Obstetrics, Gynecology and Reproductive Sciences, and Molecular Genetics and Developmental Biology, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Tony M. Plant Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Vincent Prevot Laboratory of Development and Plasticity of the Neuroendocrine Brain, Jean-Pierre Aubert Research Center, School of Medicine, University of Lille, Lille, France

Gail S. Prins Department of Urology, University of Illinois at Chicago, Chicago, IL, USA

Aleksandar Rajkovic Departments of Obstetrics, Gynecology and Reproductive Sciences, Human Genetics, and Pathology, University of Pittsburgh, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Cyril Ramathal Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA, USA, Department of Obstetrics and Gynecology, Stanford University, Stanford, CA, USA

Renee A. Reijo Pera Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA, USA, Department of Obstetrics and Gynecology, Stanford University, Stanford, CA, USA

JoAnne S. Richards Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Emilie F. Rissman Department of Biochemistry and Molecular Genetics, Program in Neuroscience, University of Virginia School of Medicine, Charlottesville, VA, USA

Bernard Robaire Departments of Pharmacology and Therapeutics, and Obstetrics and Gynecology, McGill University, Montréal, QC, Canada

Mark S. Roberson Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Sarah A. Robertson Robinson Research Institute and School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia

John A. Russell Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK

Yoel Sadovsky Department of Obstetrics, Gynecology, and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, PA, USA

Masayuki Shimada Laboratory of Reproductive Endocrinology, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

Valerie Simonneaux Institut des Neurosciences Cellulaires et Intégratives, Strasbourg, France

Lee B. Smith MRC Centre for Reproductive Health, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK

Richard L. Stouffer Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA

Susan S. Suarez Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Melissa A. Suter Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA

Amanda Swain Division of Cancer Biology, Institute of Cancer Research, London, UK

Manuel Tena-Sempere Department of Cell Biology, Physiology and Immunology, University of Córdoba, Córdoba, Spain, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Spain, Instituto Maimónides de Investigaciones Biomédicas (IMIBIC)/Hospital Universitario Reina Sofia, Córdoba, Spain

Ei Terasawa Department of Pediatrics, School of Medicine, and Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI, USA

Kent L. Thornburg Department of Medicine, Center for Developmental Health, Knight Cardiovascular Institute, Oregon Health and Science University, Portland, OR, USA

Paul Le Tissier Center for Integrative Physiology, University of Edinburgh, Edinburgh, UK

Kiyotaka Toshimori Department of Reproductive Biology and Medicine and Department of Histology, Chiba University Graduate School of Medicine, Chuo-ku, Chiba, Japan

Hanna Valli Departments of Obstetrics, Gynecology and Reproductive Sciences, and Molecular Genetics and Developmental Biology, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

William H. Walker Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Wipawee Winuthayanon Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

Selma Feldman Witchel Department of Pediatrics, University of Pittsburgh School of Medicine, and Children's Hospital of Pittsburgh, Univeristy of Pittsburgh Medical Center, Pittsburgh, PA, USA

Larry J. Young Department of Psychiatry and Behavioral Sciences, Center for Translational Social Neuroscience, and Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA

Anthony J. Zeleznik Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, and Magee-Womens Research Institute, Pittsburgh, PA, USA

Introduction

This, the fourth edition of "Knobil and Neill's Physiology of Reproduction," is a major departure from its first three editions by having Editors in Chief not previously involved in managing the production of this work. Before his death in the year 2000, Ernst Knobil and I had made plans for the transition to an Editor in Chief who had been trained at the postdoctoral level in one of our laboratories. Dr Tony M. Plant was chosen from among several excellent candidates who had the requisite characteristics needed to continue production of a work that would have the same high standards we hoped to have maintained in the earlier editions. I first met Tony in the early 1970s while I was a young faculty member at Emory University in Atlanta, Georgia, and he was employed at the Georgia Mental Health Institute also located in Atlanta. I received a letter from Ernst Knobil inquiring about my impression of Tony, who had applied for a postdoctoral fellowship in Ernst's laboratory where I had trained as a fellow but had recently relocated to Emory. My reply was a strong endorsement of Tony, which was borne out in subsequent years. Tony Plant chose Dr Tony Zeleznik as a Co-Editor in Chief to share with him the demanding tasks of editorship requiring a broad base of information in the field and a high level of academic rigor. My perusal of the list of authors and their chapter titles suggests that the information presented in this edition will rival in quality that presented in the first three editions, i.e., the work is a modern synthesis of reproductive physiology of mammals at the molecular, cellular, and organismic levels. This is not surprising given the excellent past records of the two Editors in Chief.

Ernst Knobil and I believed strongly that any work claiming to be comprehensive and hewing to the highest principles of scholarship must include a summary of the founding and the founders of the field. Because Dr Roy O. Greep was one of the leading founders of the field, we requested that he provide that information together with an overview of the future of the field in his Foreword for the first edition in 1988. Thus, the original Foreword had the great strength of being written by one of the last surviving founders in the field, and thus avoided the greatest weakness of all historical analysis, namely that history is defined as what professional historians define it to be.

A new Foreword has been prepared by Dr M. Susan Smith to address, in part, Greep's predictions of progress that might occur in the field in the succeeding years after his Foreword first appeared. Susan is a prominent leader in reproductive physiology due, for example, to her Presidency of the Endocrine Society, Directorship of the Oregon National Primate Research Center, and maintaining an National Institutes of Health (NIH)-funded laboratory running for several decades. I am pleased that her fellowship training occurred in my laboratory at Emory University during which we published the most highly cited paper of my career, of which she was the senior author.

Jimmy D. Neill

Preface

Having had the fortune of being mentored by Ernst Knobil in the early stages of our careers and serving as assistant professors in his faculty in the Department of Physiology at the University of Pittsburgh School of Medicine, we were greatly honored by the invitation to serve as Editors in Chief for the 4th edition of this book that has come to be recognized by many as the "Bible of Reproduction." At the same time, we undertook the task with considerable trepidation, being fully aware of how difficult it would be to fill the shoes of Professors Knobil and Neill, who, with their utmost attention to scientific rigor, had, together or individually, guided the previous three editions.

Given the foregoing challenge, we began by evaluating whether the structure and content of the previous edition needed to be refocused. It quickly became obvious that this was indeed the case because of the rapidly growing use of "omics" approaches in most, if not all, areas of reproductive biology. In this regard, it is noteworthy that the first edition of this text was published in 1988, a year before the first use of the "gene knockout" approach in mice was described. Over the course of the 25-year history of this text, the ability to delete or modify individual genes has increasingly and significantly added to the classical armamentarium of organ ablation/hormone replacement paradigms previously available to investigators studying reproduction. In addition, the application of genetics/genomics to elucidate the etiology of human disease has increased in parallel to the use of transgenesis in experimental animals. Collectively, these powerful approaches have provided new, and often serendipitous, information on the regulation of reproduction. For example, our view of the neuroendocrine control of the hypothalamic–pituitary–gonadal axis is now completely dominated by kisspeptin. This hypothalamic peptide, which, in the context of reproduction, was unheard of before 2003, surfaced because mutations of its receptor, then known as GPR54, were identified in individuals with delayed puberty.

Accordingly, our authors were confronted with the need to integrate information gleaned from "classical" whole animal studies with results generated by contemporary "omics" approaches in order to provide a holistic overview of reproductive processes. In keeping with this approach, we encouraged authors to discuss the historical underpinnings of their respective fields and to identify the burning questions that remain to be addressed.

In this 4th edition, Volume 1 focuses on basic processes of male and female reproduction while the thrust of Volume 2 is on the physiological control systems that govern reproductive processes, including those regulating sexual behavior. A chapter on meiosis has been included for the first time, and discussions of epigenetic mechanisms that are emerging as important regulators of reproduction, as well as fetal origins of adult diseases, have been expanded. New chapters on spermatogonial stem cells and hormone signaling in the testis that provide contemporary views of the control of testicular function replace the more classical approach to the control of spermatogenesis that was presented in prior editions of the text. We have also expanded the discussion of the regulation of reproductive behavior, which, having moved far away from its origins in ethology, is now dominated by genomic, epigenetic, cellular, and molecular approaches to the understanding of sexual motivation, partner preference, and parental behavior. For those chapters that have been updated, we are confident that these new versions present the most current discussion of the subject material.

Such restructuring of this book would not have been possible without the enthusiasm and commitment of our section editors, to whom we are greatly indebted. A feature of the 3rd edition that has been maintained is the inclusion of the Foreword by Professor Roy O. Greep that was written for the 1st edition, published in 1988. As recognized by Professor Neill, the Editor in Chief of the previous edition, Greep's account of the future of research on the physiology of reproduction continues to remain remarkably relevant, now 26 years after it was written. We are pleased to include a second Foreword that was written by Dr M. Susan Smith, who worked with Jimmy Neill as a postdoctoral scholar and was also a member of Ernst Knobil's Department of Physiology at the University of Pittsburgh School of Medicine. Dr Smith's broad understanding of both whole animal and molecular approaches used in the study of reproductive processes makes her uniquely qualified to comment on how the study of reproduction has progressed since Greep's original Foreword in 1988, as well as to identify the challenges to be met in future years.

This 4th edition of *Knobil and Neill's Physiology of Reproduction* will be the last available in a print version. While our purpose here is not to debate the loss of hard copy, electronic publishing will enable, on the one hand, the addition of new chapters covering important emerging fields and, on the other, the replacement or revision of outdated chapters, both with relative ease. In this way, we anticipate that the *Physiology of Reproduction* will continue to be highly contemporary and comprehensive and therefore remain, for the future, the authoritative text of the field.

It is our expectation that this 4th edition of *Knobil and Neill's Physiology of Reproduction* will continue to kindle the importance of physiology in the understanding of reproductive processes and, in the words of Neill and Knobil in their preface to the first edition, be "useful to all serious students of reproductive physiology be they scientists, teachers or physicians."

Tony M. Plant
Anthony J. Zeleznik

Foreword* by Roy O. Greep

I am pleased and honored to have been asked to prepare the Foreword to this volume of work depicting the progress in research on the physiology of reproduction as well as the resulting gains in understanding made over the past few years. The expertise that is represented by the numerous contributors to this work is so impressive that I am humbled even to contemplate adding anything of note. It is only by virtue of having personally witnessed a very large segment of twentieth century research on reproduction that I am emboldened to reflect on the byways and the trailblazings that have brought this field to its present proud state of enlightenment with regard to the long sought-after means of controlling the procreative process in humankind. Clearly, there are many important and knotty problems yet to be resolved, but the pace of progress over the past several years has quickened to the extent that one is left in expectant wonderment as to where and when the next revolutionizing development will occur.

The experimental method of studying reproduction was initiated in 1849 with Berthold's discovery of a blood-borne activity that came from the testis and stimulated growth of distant organs such as the comb and wattles. In so doing he utilized one of the most fundamental means of demonstrating the function of an endocrine organ, namely, surgical removal to determine what deficiencies follow, coupled with implantation or transplantation to ascertain whether the deficiencies were repaired. At that time it was not possible to take the next step, namely, preparation of an active extract of the testes, because nothing was known about the nature of the bioactivity. Forty years later, Brown-Séquard claimed to have prepared an active extract of dog testes; however, as is well known, his enthusiastic claims for restoration of his own sexual activity at an advanced age were not substantiated. Actually, these simple means of studying reproductive physiology persisted well into the twentieth century, including the studies of such pioneering stalwarts as Marshall, Heape, Prenant, Bouin, Ancel, Loeb, Cushing, and Aschner. Observations otherwise were limited to cyclic and seasonal changes in sexual behavior among common laboratory and small domestic animals. This type of eyeball research remained in vogue through the early 1920s and overlapped the extension of visualization to the microscopic level. The latter revealed, for the first time, the precise timing of events in the ovarian cycle through microscopically observable cellular changes in the vaginal fluid. My point in mentioning these early studies is to emphasize that although the tools and techniques were inordinately primitive by present standards, the results established a firm base of knowledge on which to build.

The study of cyclic changes in the vaginal smear in rats and the findings of estrogenic activity in follicular fluid during the early 1920s led to an explosion of interest in the study of reproduction. The field was fortunate in attracting to its ranks a small band of exceedingly able biologists and biochemists who, in 1932, were to become authors of the classic first edition compendium, Sex and Internal Secretions, a volume overwhelmingly devoted to reproductive endocrinology. It was this landmark of progress that finally gave propriety to the study of reproduction and put it on a par with the study of other major bodily systems. Incredible as it may seem, it was only a decade earlier that a distinguished panel of the National Research Council had declared that sex research was not a fitting topic for scientific study.

Lest our pride in today's spectacular pace of progress unduly bedazzle the mind, it should not be overlooked that the developments recorded in the 10-year span from 1926 to 1936 may never be equaled. Among those monumental achievements, all of the native sex steroid hormones were brought to light, their structures were determined, their functions were defined, and they were made available in pure form for research and therapy. Similarly, all of the pituitary, placental, and urinary tropic hormones were identified, and their functions were defined. Like today's competition for priority rights, publicity, and potential financial gain, these earlier periods also were times for intense rivalries, but rarely with prospects for financial rewards. It would be difficult to overstate the boost that was given to basic and clinical research in reproduction as a result of the availability of estradiol-17β, testosterone, and progesterone in pure form and of known potency. The replacement of

*This Foreword by Roy O. Greep is reprinted from the 1988 (first) 1994 (second) and 2006 (third) editions of this work (E. Knobil and J. D. Neill, The Physiology of Reproduction. Raven Press, New York. Copyright Elsevier). This now-deceased author wrote a remarkably prescient account of the future of research in the field that remains as relevant today as it did in the first three editions.

homemade extracts and such elastic entities as rat units, mouse units, capon units, and so forth, with micrograms of pure hormone was revolutionizing and allowed the study of reproduction on a quantitative basis.

Prior to World War II the thrust of research on reproduction dealt predominantly with the steroid hormones. This was the heyday of steroid biochemistry. After World War II the emphasis shifted to the protein and peptide hormones, where it still remains strong. This prolonged and difficult effort yielded many biochemical triumphs. Most notable among these were the isolation of the pituitary, placental, and urinary gonadotropins, as well as the determination of their primary structure as glycoproteins comprised of two dissimilar and covalently bonded subunits, the isolation and synthesis of the gonadotropin-releasing hormone (GnRH) of hypothalamic origin, and the isolation and structural characterization of relaxin.

The availability of pure protein and polypeptide hormones made possible the production of hormone-specific antibodies as well as the application of immunological techniques to the study of reproduction. An outcome of great consequence was the development of radioimmunoassay as the new means of measuring all of the hormones relating to reproduction. The sensitivity of this new technique was so great that it made possible, for the first time, the measurement of all these hormones in the body fluids. It had the further distinct advantage of requiring such a small amount of fluid that the monitoring of blood levels of the hormones of reproduction could be done throughout an estrous or menstrual cycle by close serial sampling. This revealed still another and most unexpected finding, the pulsatile pattern of secretion.

Identifying the homeostatic mechanism(s) responsible for maintaining a steady state in various physiologic systems of the body has been fraught with many challenging problems, but these pale in comparison with the difficulties encountered in trying to elucidate the mechanisms maintaining a constantly changing system, a characteristic of the reproductive system of female mammals. The earliest piece of evidence suggested the existence of a "push–pull" mechanism that later came to be known as negative feedback. It was based on the demonstration that an estrogenic extract administered to immature rats would maintain the ovaries in an infantile state. This was quickly followed by conclusive evidence that estrogen acted to inhibit pituitary follicle-stimulating hormone (FSH) stimulation of follicular growth and maturation; however, the effect on luteinizing hormone (LH), ovulation, and luteinization remained unsettled. Gaps continued to exist in all proposed explanations of reproductive cycles. None of these explanations took into account the influence of photoperiodicity on seasonal breeders, nor did they account for the role of the stimulus of mating in nonspontaneous ovulators. Following the discovery of the hypothalamic control of pituitary function, estrogen was shown to exert its action on both the pituitary and the hypothalamus; however, the problem of accounting for cyclicity remained. Adding to the complexity, radioimmunoassay revealed an unexpectedly high level of blood estrogen just prior to ovulation, an event not in keeping with the negative feedback concept.

Finally, after many years of searching for a way out of this frustrating situation, a glimmer of light appeared at the end of this long dark tunnel—light that soon turned to brilliance. In 1969, Goding and associates found that the administration of large doses of estrogen to ewes at the time of estrus did not block, but instead entrained, ovulation. Shortly thereafter, in more elaborate examination of the relationship of blood estrogen levels and ovulation in rhesus monkeys in Knobil's laboratory, it was revealed that elevated estrogen levels preceded and appeared to trigger ovulation. On further examination, Knobil and colleagues found that when blood estrogen reached a critical level, the feedback mechanism switched from a negative to a positive, or stimulative, action. This utterly new finding greatly advanced our understanding of the endocrine mechanism governing reproductive cycles. There still remain, however, some uncertainties: Why does the switch in feedback action occur; to what extent and at what stage of the cycle does estrogen act at the level of the pituitary or the hypothalamus, or both; and lastly, what role, if any, do the ovarian peptides, especially inhibin, play in controlling reproductive cycles?

The progress of research on reproduction has been chronicled in numerous review articles by individual authors. Many have appeared in Recent Progress in Hormone Research, Volumes 1–42. Other major sources include the multiple editions of such titles as: Marshall's Physiology of Reproduction, Fourth Edition (1990); Sex and Internal Secretions, whose third and last edition was issued in 1961; two volumes on the Female Reproductive System (1973), and one on the Male Reproductive System (1975) in Section 7 of the Handbook of Physiology, published by the American Physiological Society; and four serial volumes on reproductive physiology in the International Review of Physiology, the last one being issued in 1983. The present volume will provide comprehensive coverage and meet the current needs of the field of reproductive physiology, a field that is rapidly gathering momentum from the application of new and highly sophisticated tools and techniques.

In viewing the vast literature dealing with research on the male and female reproductive systems and considering the rate at which it is accumulating, one might ask whether this staggering proliferation of books and articles is essential to progress; the answer is an emphatic "Yes!" The yardstick by which progress is measured

in this or any other field is not in the number of articles published or the amount of financial support but in improved understanding. Such gains are generally marked by sharp peaks at indeterminate intervals, separated by avalanches of incremental gains, as recorded in an ever-growing list of journals. The point to remember is that without this persistent chipping away at a major problem there would be no solutions and no quantum leaps forward. In research very little comes from out of the blue. Part of the driving force in research is its adventuresome nature and ever-present possibility that one's efforts will pay off in an important manner. It may not be entirely fair, but in research (as in most human activities), the spoils go to the victor in the form of kudos, prizes, awards, public attention, and, increasingly in the present technological age, monetary gains—sometimes of great magnitude. What effect this latter may have, if any, on the long-cherished sanctity of science has not been determined, but it has become a matter of concern.

This volume bears the title The Physiology of Reproduction. Physiology, by traditional consensus, is that branch of science, which studies the functions of a living organism or any of its parts and includes the basic underlying processes. It will be understood that most of the studies reviewed here will be based more on holistic research than on research at the submicroscopic or molecular level. It is unfortunate that the excitement generated by recent fantastic advances in molecular biology and development has tended to downgrade the value of whole-animal research, and physiology in particular is sometimes looked upon as passé. Actually, the two categories of research are complementary, and both are essential for maximum advancement of knowledge. Whole-animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated.

In the same vein, no one immersed in reproductive endocrinology can be unaware of the current tendency to regard research at the molecular level as representative of exceptional scientific talent. This is a common consequence of the opening of a new arena of investigation. I recall an incident that happened at a scientific meeting back in the 1930s. The first three papers in a session chaired by an eminent embryologist were on endocrine topics—mine was the third. That being ended, the chairman took pains to assure the audience that the meeting could now turn to considerations of more fundamental nature. One of the other three papers was given by Herbert M. Evans, who bristled noticeably but held his fire. There was also an earlier period when one either worked on steroid biochemistry or something of lesser appeal like biology. Anyone who remembers the 1950s will recall a flash in the pan ignited by cybernetics, a study of automatic control systems both neural and physical. The gurus of cybernetics captured the attention of the press and of audiences throughout the land, but eventually this obsession suffered the fate of other passing preoccupations. My own observation is that the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make the observations and to read out results that are often quite free of extraneous variables. Toward the obverse situation, one's dependence on an extensive background of experience and physiological increases as does the unavoidable complex of in vivo variables that must be taken into account. In either case we have today the availability of far more diverse approaches to a given problem in any field of biomedical research than has ever existed before. In Berthold's day there was only one experimental method available; today's number is untold but is probably in the hundreds, perhaps thousands. This is an exceedingly promising situation and one to which investigators of all persuasions must adjust. Open minds will experience exhilaration over substantive achievements at any point on this observational spectrum.

One of the major factors influencing research on reproduction has been the availability of funds or lack thereof. Prior to the institution of federal funding (i.e., prior to the middle of the twentieth century), reproductive research was sparsely supported by university departmental funds, industry, small grants from the Committee for Research in Problems of Sex within the National Research Council, and some aid from the Rockefeller Foundation. The National Institutes of Health were slow in providing significant support of research on reproduction because of restrictions on the support of work related in any way to birth control. This occurred despite the simultaneous postwar baby boom. What kept research afloat during this critical period was major support by the Ford Foundation plus lesser contributions by other major foundations. It was not until the establishment in 1968 of the Center for Population Research in the NICHD that major governmental funding in this area became available, but the boost was short-lived. As a result of the imposition of fiscal restraints in the early 1970s, federal support dwindled and has remained at a minimal level ever since. Support from all sources is woefully incommensurate with the distressing expansion of the human population and the need for safe, effective, economical, and readily available means of limiting human fertility.

The physiology of reproduction is predominantly under hormonal control. The first essential step in studying reproduction was identification of the hormones involved and the functions they serve. This having been accomplished, efforts turned to a detailed analysis as to how hormones act within the body. During the 1980s there has been a rising tide of interest

in the binding of steroid, protein, or peptide hormones to receptors on specific target cells. Much effort is currently being directed toward the isolation and chemical characterization of these receptors. They are known to be composed of a protein or proteins, and some information has already been gained as to their partial or provisional structure. This, however, is only a preliminary step in the complex process whereby hormone action results in an end response such as growth, secretion of a target cell hormone, or altered behavior. The curtain has already been raised on the climatic and final chapter of the story on how hormones act. This involves linkage of the hormone–receptor complex with the nuclear genetic apparatus leading through a now well-defined series of processes to the manifestation of a physiological response in the living organism. Genes that bring about the expression of certain hormonal signals are being isolated, modified, transferred between species, and also inserted into bacteria where they direct the biosynthesis of specific hormones in large quantity. Thus genes are being manipulated in ways that raise the potential of altering the reproductive process. It is largely as a result of developments in endocrinology at the molecular level that bewildering possibilities loom on the horizons of reproductive research—they are within reach; they are science, not fiction; and they stagger the imagination.

It being granted that nothing succeeds like success, then the new edition of this highly successful two-volume compendium on The Physiology of Reproduction is destined for an illustrious fate. This second edition will maintain the same high standards of the first and again fulfill an existing need in a field that is experiencing rapid growth and exhilarating progress. Like the first edition, this one will provide a critical assessment of the state of the art in every aspect of research on the physiology of reproduction by eminent authorities.

In the years intervening between this edition and the last, notable changes have taken place in the study of reproduction. These stem largely from major advances in technology. Remarkable new instruments, techniques, and methods have enabled investigators to probe ever deeper into the interaction between hormones and genes, thereby eliciting in vivo responses. New parameters are being added to the target tissues of the classical reproductive hormones as revealed by the presence of receptor sites in tissues, the physiologic significance of which often remains tantalizingly obscure. Similarly, newly identified substances of endocrine or paracrine nature are being added to this domain of research with persisting frequency. Some of these substances—the endothelins, interleukins, activins, inhibins, and prorenin, to name a few—also exhibit a puzzling array of effects on extraneous tissues. Their study is being aided by the fact that their structure is known and, though rare, they are available.

Great strides are also being made in many other aspects of research on reproduction. Much work is being done on the structure of receptors and the loci of binding sites on segments of the folded gonadotropic molecules. A full-scale effort is underway seeking an elucidation of the neural mechanism underlying pulsatile secretion. Neuroendocrinologists are closing in on an elusive pulse generator located in the central nervous system. This looms as another landmark discovery in reproductive biology.

Research on reproduction is flourishing and the future appears bright. The taboos are gone. All aspects of the reproductive process are an open book. One area that has taken a quantum leap forward is the clinical application of an important body of relevant new knowledge gained in both basic and clinical spheres. Expanded opportunities have been opened by greatly improved diagnostic procedures, more effective treatment of disorders, and new methods of controlling fertility. Contributing greatly to this explosive development is the dissemination of information on reproductive matters to the lay public by the mass media. Concerned individuals have been made aware of the existing new means of manipulating the male and female reproductive systems for enhancement or inhibition of fertility. The joys and comforts that accrue respectively to these opposing modes of fertility control have enriched the lives of a grateful public. To that end I may note that it was by virtue of these frontier reproductive measures that my own progeny includes a new grandson and namesake.

Roy O. Greep

Foreword by M. Susan Smith

I am truly honored to write the Foreword for the 4th edition of this book; it is a humbling task to follow in the footsteps of Professor Roy O. Greep, whose Foreword is herein reprinted from the 1st, 2nd, and 3rd editions of this work. His remarkable account of the field of the physiology of reproduction was written 26 years ago, yet as you read it, you will develop an overwhelming appreciation for how fortunate we were to have Professor Greep as one of the foremost leaders in our field. It is especially gratifying for me to be associated with this great work, since my scientific lineage is part of the Greep/Knobil/Neill 'family', having trained with Professor Neill and served as a faculty member in Professor Knobil's Department of Physiology at the University of Pittsburgh.

Since the publication of the 3rd edition of this book in 2006, there continues to be remarkable progress in the physiology of reproduction, reflecting the use of advanced technologies, such as those spurred by contemporary genetic and genomic approaches. However, the challenge today is to understand how this genetic and molecular information is integrated into the manifestation of a physiological response. Greep's thoughts on this are still prescient today, "Actually, the two categories of research are complimentary…." "Whole animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated."

Professor Greep issued a challenge in his Foreword: "Developments recorded in the ten-year span from 1926 to 1936 may never be equaled." I posit that the 10-year span from 2004 to 2014 was, at the very least, equally important, as we have made a paradigm shift in how we conduct our science. In the more traditional way of science, experiments focused on understanding control systems; this then led to the discovery of new molecules. Today, in contrast, the use of massively parallel sequencing (exome sequencing, ChiP-seq, and RNA-seq) allows us to identify all the players, even though we may have no idea of their function. We then "reverse engineer" results from these studies to discover where a particular molecule is produced, how its production is regulated, and what its function is. These sequencing techniques were used in the groundbreaking studies that signaled the important role of kisspeptin and neurokinin B in regulating the pituitary–gonadal axis; subsequent studies located their sites of production in the hypothalamus and identified their critical function in controlling GnRH neuronal activity. All of these sequencing techniques generate massive amounts of data and necessitate application of the extensive bioinformatics infrastructure that goes with managing large databases. Greep was right when he stated, "the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make observations and to read out results that are often quite free of extraneous variables." As an example of the power of these databases that are available to the scientific community, there is one that identifies all the transcribed genes in the ovary of the nonhuman primate and maps changes in their individual activities throughout the duration of a menstrual cycle and into early pregnancy. Not too long ago it would have been impossible to imagine being able to follow the activity of one gene through such a time course. Importantly, these databases can be used for comparative analyses among species and should provide enlightenment about the evolution of different approaches to reproduction.

The material in the chapters of this 4th edition reflects advances that have been made at all levels of the hypothalamic–pituitary–gonadal axis and the reproductive tract; many of these advances have been made possible by the new tools available to scientists. The most notable is the discovery of kisspeptin in the hypothalamus that fundamentally changed our concept of the control of GnRH neurons and has expanded our knowledge of the neural networks that govern reproductive function. Significant advances have also been made in our understanding of how G-protein coupled receptors function; such as the insight gained by the discovery of GnRH receptor misfolding that results in a loss of trafficking of the receptor to the plasma membrane and, thus, functionality, a deficit that can be overcome by artificial chaperones. New areas of research have come forth, such as oncofertility, a term coined to signify the restoration or maintenance of fertility in cancer patients whose gonadal function is diminished or lost due to the side effects of their radiation treatment or chemotherapy. Studies using new techniques, such as 3-dimensional culture of follicles in an extracellular matrix and differentiation of gamete stem cells, have provided new insights into the processes controlling folliculogenesis and gametogenesis, with translational

opportunities for promoting or controlling fertility. In addition, there is an explosion of interest in stem cell biology, a field that was rooted in reproductive research and the practice of in vitro fertilization. There is also a new appreciation for the role of the environment in fetal development and this has led to the birth of a new area of medicine known as DOHAD (Developmental Origins of Health and Disease). Maternal under- and overnutrition both lead to epigenetic changes in the fetus that can have long-lasting consequences in the adult. Similarly, exciting new research shows environmental influences on epigenetic modifications of DNA in sperm that may result in paternal transmission of disease risk.

When considering all of this new research, some caveats need to be kept in mind. There appear to be significant species differences in a number of reproductive processes, including puberty, ovarian cyclity, and parturition, yet current research focuses primarily on the transgenic mouse model because of the ability to manipulate the expression of specific genes. Therefore, many of our current ideas about the control systems regulating reproductive processes are "mouse-centric" and may not be directly applicable to other species. This may create problems as we look to translate this information into new therapeutics for use in human and veterinary medicine.

What new advances can we look forward to in the near future? New technologies, such as optogenetic/pharmacogenetic tools, designer receptors, and advanced imaging, will likely contribute to the making of another "best decade" from 2014 to 2024. But it will be important to develop better methods, with increased specificity and sensitivity, for measuring hormones and other substances in the blood. There are also growing concerns over the use of sex steroids in clinical therapies. As a result, there is considerable interest in developing "nonhormonal", i.e., nonsteroidal, selective therapies that act at the local or intracellular levels.

Future studies must also focus on the still many critical unanswered questions in the physiology of reproduction. Just in the area of neural regulation of reproduction alone, there are numerous examples. Many external signals modulate reproductive function, such as stress, endocrine disrupters, diet, photoperiod, and pheromones. Yet we still do not know how these external signals are transmitted to directly alter kisspeptin or GnRH neuronal activity and thus, reproductive function. Perhaps, Greep's assertion that "The physiology of reproduction is predominantly under hormonal control," needs to be expanded to include nonhormonal factors that are important modulators. Another mystery is what heralds the onset of puberty. Detailed information is known about the regulation of kisspeptin gene expression and its upregulation at the time of puberty, but the specific signals that bring about these epigenetic changes are still completely unknown. Similarly, although neuroendocrinologists are closing in on the GnRH pulse generator located in the central nervous system, the actual mechanisms involved in pulse generation remain elusive. Greep recognized that "This looms as another landmark discovery in reproductive biology." The processes involved in the positive feedback effects of estrogen on gonadotropin secretion also remain a mystery. With the current recognition that there are likely species differences in these processes, negative and positive feedback of estrogen might be achieved through two different populations of kisspeptin neurons (rodent model) or two different populations of GnRH neurons (nonhuman primate model).

While the future for research in reproduction appears bright, with more landmark discoveries in the making, there is also cause for concern. Funding for basic research in reproduction at the federal level is declining, as the emphasis shifts to translational research relevant to human health and disease. In the bench-to-bedside continuum of research, it is critical to keep "bench" in the equation. There are still many areas in reproduction where we do not understand the basic underlying controlling mechanisms, making it difficult to devise therapeutics. Studies of various species will also be harder to support. This is regrettable, since comparative studies have revealed differences in how reproduction is regulated, and this collective information may be critical to solving long sought after questions, such as, what constitutes the GnRH pulse generator, how is puberty initiated, how is parturition initiated, and what is the impact of environmental factors on germ cells. A good example of the value of studying various species is the seasonal regulation of reproduction that led to a focus on photoperiod and how time-of-day signals are transmitted to GnRH neurons. These studies were instrumental in advancing the field of chronobiology to the recognition today that all cells appear to have clock genes that regulate their function. It is also regrettable that there are still constraints on contraception research even though the link between the ability to control fertility and the economic development of a country is well established. Finally, in this age of "big data," it is well to remember that analysis of large databases can detect correlations, but it cannot determine whether the correlations are meaningful or provide information about causality or mechanisms. This is the province of basic research: to discover the critical information about control systems that then provides the underpinnings for translational research and the development of new human therapeutics.

M. Susan Smith
Division of Diabetes, Obesity and Metabolism, Oregon National Primate Research Center, and Oregon Health and Science University, Beaverton OR, USA

CHAPTER

1

Mammalian Meiosis

Paula E. Cohen, J. Kim Holloway

Department of Biomedical Sciences, Cornell University, Ithaca, NY, USA

INTRODUCTION

Basic Overview of Meiosis and Comparison with Mitosis

Mitosis and meiosis are the two types of cellular division that are common amongst eukaryotic organisms. While mitosis involves the faithful replication of cellular contents, including DNA, to produce two daughter cells of identical genetic and cellular composition, meiosis is confined only to those cells that participate in sexual reproduction: the germ cells (or gametes) in mammals. The goal of meiosis, in contrast to mitosis, is to produce gametes that have half the content of DNA within them so that, upon fertilization, the new zygote will contain the full complement of genetic information. This is achieved by virtue of the fact that each cell in a diploid organism contains two copies of every chromosome, each chromosome being referred to as "n" and a diploid organism therefore being referred to as a "2n"-containing organism. The two copies (or homologs), one of every chromosome, are provided by two parents: one maternal copy of every chromosome (chromosome 1, chromosome 2, chromosome 3, etc., up to chromosome 22, plus one X chromosome in humans) and one paternal copy of every chromosome (chromosomes 1 to 22, plus either an X chromosome or a Y chromosome in humans). Figure 1.1 provides a cartoon graphic depicting an imaginary meiotic species containing just four chromosomes: two paternal and two maternal. In this example, the long chromosomes (black and gray) are chromosome 1 homologs and the shorter chromosomes (white and off-white) are chromosome 2 homologs. Meiosis in germ cells begins with one round of premeiotic DNA replication to achieve "4c" DNA content, with each chromosome (each maternal "n" and each paternal "n" chromosome) now consisting of a pair of sister chromatids (each termed "c") that are tethered together through the process of cohesion. In Figure 1.1, therefore, the premeiotic replication results in the same four chromosomes, but now each chromosome consists of two chromatids, making a total of four chromatids for chromosome 1 and four chromatids for chromosome 2 (hence, two black chromosome 1 chromatids, two gray chromosome 1 chromatids, etc.).

Following DNA replication, cells enter meiosis, which consists of two consecutive rounds of division producing haploid gametes; these two rounds encompass meiosis I and meiosis II, respectively. In meiosis I, homologous chromosomes pair during prophase I (Figure 1.1: black and gray chromosomes pair, and white and off-white chromosomes pair) and then separate at metaphase I, resulting in the formation of two daughter cells each containing just one of the homologs for each chromosome (Figure 1.1: black or gray, and white or off-white). The meiosis I division is referred to as "reductional" because it results in daughter cells that contain only half the number of chromosomes that existed in the originating parent cell (although the daughter cells' chromosomes each now consist of paired chromatids). As will be discussed in detail in this chapter, some portions of these homologous chromosomes are exchanged in a process known as homologous recombination, and this is largely responsible for keeping the homologs together until metaphase I. Once the meiosis I daughter cells are formed, these can then undergo meiosis II, in which the paired sister chromatids now separate. This division is called "equational" because it results in the same overall number of chromosomes in the daughter cells as in the parental cell at the end of meiosis I. As can be seen in Figure 1.1, there are distinct differences in the number of products that arise following meiosis I and meiosis II in male and female germ cells, and these differences will be discussed in detail here.

The unique and defining features of meiosis occur during meiosis I, which consists of prophase I, metaphase I,

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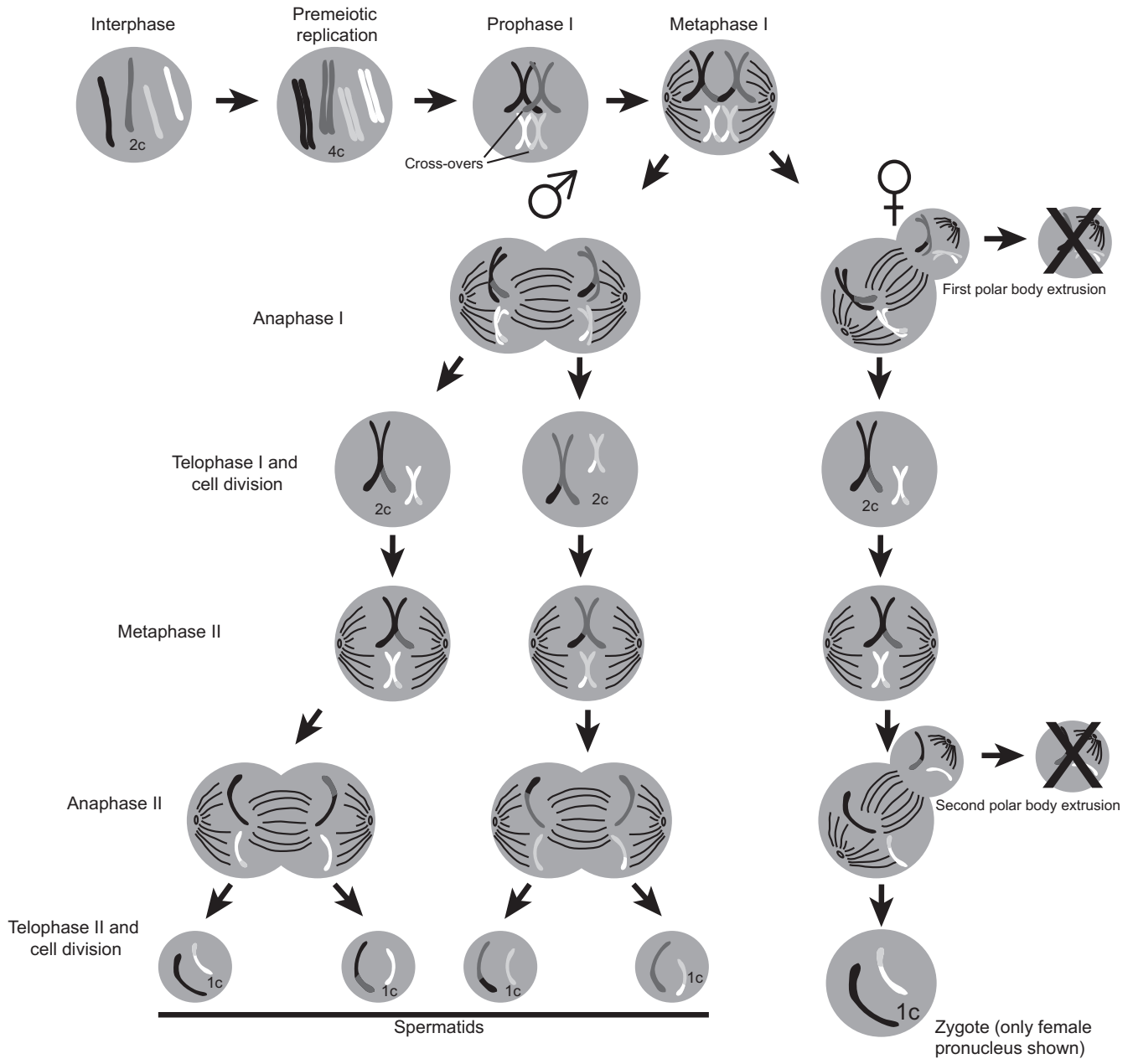

FIGURE 1.1 Stages of meiosis. Prior to the start of meiosis, the chromosomes are copied in a stage of premeiotic replication, taking the DNA compliment from 2c to 4c. In other words, there are still two copies of each chromosome (2n), but each of them has two sister chromatids (2c each, for a total of 4c). Upon entry into meiotic prophase I, the homologous chromosome pairs undergo two important events, synapsis and recombination. They then enter metaphase I, where the homologs line up on the metaphase plate and are attached to the meiotic spindle. Homologous chromosomes are separated during anaphase I, and the separation of the nuclei is completed during telophase I. Cell division (cytokinesis) then follows to produce two daughter cells with a chromosome compliment of 1n each, but with each chromosome still containing two sister chromatids (2c). In males, this process divides the cellular components equally between both daughter cells, while in females, half of the genetic complement is retained in the oocyte, and half becomes encapsulated into a smaller structure known as the first polar body. In this case, the asymmetrical division results in the majority of the cytoplasm and cell organelles dividing into the oocyte, with very little getting passed to the polar body. The second meiotic division is much more like a mitotic division, where the two sister chromatids are separated during metaphase II, anaphase II, and telophase II, resulting in haploid (1n and 1c) daughter gametes. Again, in the females, one of the products of this division is the second polar body, and it contains the second half of the genetic complement of the second division.

anaphase I, and telophase I (Figure 1.1), during which maternal and paternal homologous chromosomes come together and pair in a process known as synapsis, and then exchange genetic information through crossing over via the process of recombination. As will be described in this chapter, synapsis and recombination serve a single purpose: to keep homologous chromosomes tightly associated with each other so that they may then be

segregated equally to daughter cells. These daughter cells, each containing just one of the originating homologous chromosomes, then enter meiosis II. Meiosis II, consisting of prophase II, metaphase II, anaphase II, and telophase II, resembles more of a mitotic (reductional) division. Mitosis is the process by which cells replicate their DNA from 2c to 4c (but still 2n), and then divide the nucleus in half, separating their sister chromatids (and, thus, their DNA) equally into two daughter (diploid but still 2n) nuclei. Thus, mitosis is "cyclical" in nature, and can truly be called a cell cycle, because the resulting 2n daughter cells can be used as starting material for future cycles of DNA replication and cellular division, while meiosis is less of a cell cycle and more unidirectional considering that, once produced, gametes need to undergo fertilization in order to achieve their full diploid status and become capable of cellular proliferation through repeated mitotic cycles.

History and Discovery of Meiosis

As with so many developmental processes, meiosis was first observed and described cytologically in sea urchin eggs by the German biologist Oscar Hertwig (1849–1922) and further characterized at the chromosome level by the Belgian zoologist Edouard Van Beneden (1846–1910) in eggs from the parasitic roundworm *Ascaris*. This new nematode model, with its larger sperm size (some 10-fold larger than that of sea urchins) and abundance of synchronously developing oocytes, facilitated more precise cytological observations of chromosome movements,[1] allowing Van Beneden to observe the halving of chromosome content even before the significance of chromosomes for heredity was fully realized. It was not until the studies of August Weismann (1834–1914), however, that the significance of the two earlier studies became clear. Weismann's *germ plasm* theory held that inheritance in a multicellular organism is the product of the germ cell, or *gamete*, while the normal proliferative activity leading to the growth and development of the individual is undertaken in the somatic cell lineages. Germ cells can give rise to soma or to germ cells themselves, whereas soma can only give rise to somatic cells, a concept referred to as the *Weismann barrier* and now arguably dispelled in the era of stem cells and reprogramming. Weismann went on to demonstrate that sea urchin eggs undergo both a reductional division and an equational one, and that both were essential to provide haploid gametes that could then collectively derive a diploid daughter cell following fertilization. His work preceded the rediscovery of Mendel's work by Hugo de Vries and others in the 1900s, and these events culminated in 1911 with the discovery by Thomas Hunt Morgan of crossing over, which is the exchange of genetic information between homologous chromosomes as a result of recombination, and the raison d'être of meiosis. Hunt Morgan's landmark studies in *Drosophila melanogaster* females earned him the Nobel Prize in 1933. The term *meiosis* was coined by Farmer and Moore in 1905 to explain the process of division of germ cells, but it was the accumulation of all of the studies described above that led to full realization of the crucial role of meiosis for gamete formation.

Conservation of Meiosis across Eukaryotes

Comparative evidence seems to indicate that meiosis appeared early in eukaryotic cell history, most likely arising only once, given its high degree of conservation among many species. Even in prokaryotes, similar machineries exist for repairing recombination intermediates to those found in highly evolved species. For example, the prokaryotic single-stranded binding protein RecA, which is essential for DNA repair, has evolved to form several RecA homologs in all eukaryotes, some of which appear to be specific to meiosis, while others are shared between different forms of recombination repair. Many other meiotic proteins, such as Spo11, MutS homologs 4 and 5 (Msh4 and Msh5, respectively), and MutL homologs 1 and 3 (Mlh1 and Mlh3, respectively), all of which are described in detail in this chapter, are also functionally conserved from yeast to human.

Meiotic processes involving the establishment and maintenance of cohesion between sister chromatids, and the physical attachment of homologous chromosomes through synapsis, recombination, and crossing over, are highly conserved, if not always achieved in the same manner in all species. For example, the hallmark structural component of prophase I, the synaptonemal complex, exists in the majority of eukaryotes but may consist of different proteins (examples of synaptonemal complexes from different organisms are provided in Figure 1.2). Meiotic recombination occurs in the majority of species studied, but does not occur in male *D. melanogaster*, only in the female flies. Msh4–Msh5 proteins are major components of the meiotic crossover (CO) pathway in eukaryotes (described in detail in this chapter) but do not exist in *Saccharomyces pombe*. Similarly, MLH1 and MLH3 proteins are the major determinants of COs in many eukaryotes, but *Caenorhabditis elegans* lacks these proteins. Interestingly, however, *C. elegans* does possess MSH4 and MSH5, the binding partners for MLH1–MLH3, raising the question of whether other functionally similar proteins are present in worms to substitute for MLH1–MLH3. Therefore, in these model organisms, meiosis itself is highly conserved, but the major players may be interchangeable or the processes slightly adapted to suit each organism.

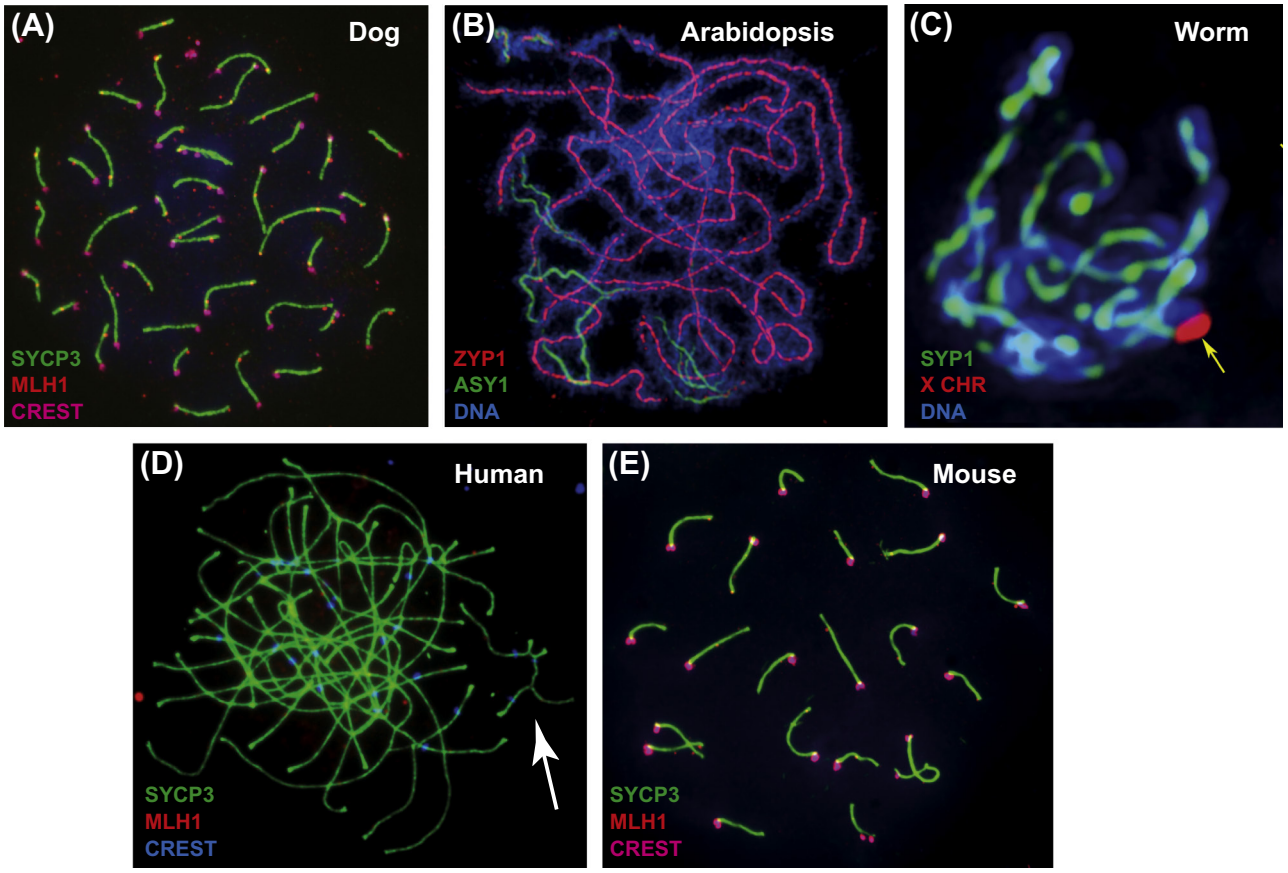

FIGURE 1.2 **Comparative meiosis across eukaryotic species.** This figure is reproduced in color in the color plate section. (A) Pachytene cell from an adult dog testis stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, pink). (B) Immunolocalization of the *Arabidopsis* synaptonemal complex transverses filament protein ZYP1 (red), polymerizing between the homologous chromosome axes marked by ASY1 (green) and the DNA loops (blue) in a late-zygotene nucleus. The ASY1 signal (green) is reduced in synapsed regions (red), and an interlock is being resolved. (C) A *C. elegans* pachytene nucleus immunostained to show that the synaptonemal complex (green) is normal and that the pairing center ends of the X chromosome are fully paired (red); *(Source: Adapted from Saito et al., PLoS Genet, 2009.)* (D) A trisomic human embryonic oocyte at the pachytene stage stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, blue). The three copies of chromosome 21 can be seen attempting to pair (arrow). (E) Mouse pachytene cell stained immunostained with SYCP3 (green), MLH1 (red), and cREST (centromere, pink).

Sexual Dimorphism and Timing of Meiosis

In mammals, one of the defining features of meiosis, and of gametogenesis in general, is that there are distinct differences in the mechanism, timing, and success of meiotic events between the sexes (reviewed by Ref. 2). The most fundamental difference lies in the number of products of meiosis, with four haploid spermatozoa being produced during meiosis in males, and only one oocyte in females, with the remaining chromosomal contents being extruded into two polar bodies, following a distinctly asymmetric division (Figure 1.1). In females, meiosis is not completed before fertilization occurs, and it is the process of sperm penetration of the oocyte cytoplasm that elicits the cascade of events that culminate in completion of the second meiotic division and extrusion of the second polar body (Figure 1.1, and see Chapter 4 for further details).

Meiosis in females begins in midgestational fetal life, with all oogonial precursors entering meiosis in a semisynchronous fashion. Oocytes undergo two periods of arrest; the first lasts weeks in mice and years in humans, and involves the entire oocyte pool. Following birth, each estrous or menstrual cycle is marked by the growth and development of a cohort of follicles, only a subset of which go on to be ovulated (one or two in humans), while the others undergo atresia. Only those oocytes that are ovulated will resume meiosis. In primates at least, the resulting diminution of the oocyte pool leads to reproductive senescence. In males, on the other hand, meiosis is initiated at or around puberty (or just after birth in rodents), and spermatogonial precursors enter meiosis in waves along the seminiferous epithelium of the testis, which reoccurs throughout the life of the individual.

For humans, perhaps the most fundamentally important difference between males and females lies in the distinct difference in meiotic error rates observed in females compared to males. This is possibly most startling in humans, in whom meiotic error rates approach 40–60% in women but remain under 3% in men (Source: U.S. Centers for Disease Control). Studies of mouse mutants have pointed to the fact that female meiosis appears to be less stringent in its regulation, providing at least one possible explanation for the differences in error rates. In this chapter, many points of divergence between male and female mammals will be discussed, some or all of which may contribute to the differences in success rates for male versus female meiosis.

Scope of the Chapter

This chapter aims to describe the main features of the meiotic process, from the earliest stages of induction during embryogenesis in females, to the last stages of meiotic cell division. Complex mechanisms are described that control meiotic induction, cohesin assembly and disassembly, prophase I events such as synapsis, silencing and meiotic recombination, and meiotic segregation. For each of these, we provide an introductory overview at the beginning of each section before delving further into the often complex molecular mechanisms involved. Environmental and aging effects on meiotic mechanisms are discussed in detail, and diseases arising from meiotic errors also feature. Overall, the chapter focuses on mammalian meiosis, with an emphasis on those stages and events that differ from mitosis, but it draws evidence from non-mammalian meiotic species for the purposes of comparison and further illustration of key regulatory processes.

INITIATION OF MEIOSIS IN MAMMALS

One of the key features that distinguishes gametogenesis in male and female mammals is the time during development in which the oogonia or spermatogonia cease their proliferative activity and enter meiosis. Early in embryonic development, the primordial germ cells (PGCs) arrive in the bipotential or indifferent gonad and begin a rapid and rampant phase of proliferation, during which time they increase in number exponentially over several days to weeks. In the mouse, for example, approximately 3000 PGCs take up residence in the bipotential gonad at around 10.5 days postcoitum (dpc) at a time when the gonadal structure has barely arisen from the intermediate mesoderm. The PGCs continue to proliferate, reaching a number of some 30,000 by 13.5 dpc,[3,4] at which point the first sign of sexual dimorphism becomes evident. The PGCs of the female stop proliferating and enter prophase of the first meiotic division, while PGCs of the male arrest in G0/G1 of the mitotic cycle and then only resume proliferation and enter meiosis after birth[5] and, most usually (although not always), at puberty.

Retinoic Acid (RA) is the Key Trigger for Meiosis in Males and Females

It has been well established that male germ cells fail to enter meiosis during fetal life as a result of inhibitory factors secreted by the developing testis, while they can be induced to undergo meiosis when co-cultured with embryonic ovaries[6] or when cultured with conditioned medium from adult testes.[7–10] Such observations gave rise to the suggestion that meiotic entry is a cell-nonautonomous event, being regulated locally, either within the gonad or by tissues in the vicinity of the developing gonad, but not by the germ cells themselves. The mesonephros, which is closely apposed to the developing gonad, was suggested as the site of "meiosis induction" when it was demonstrated that co-culture of mesonephros with fetal bipotential gonads could induce feminization, or meiotic entry.[11–13] These studies were confounded, however, by early observations showing that ectopically located germ cells (that migrate aberrantly to other regions of the embryo) spontaneously initiate meiosis without close proximity to the mesonephros,[14,15] raising the possibility that gonad-intrinsic factors may also induce meiotic entry.

Evidence for the role of vitamin A–retinol metabolite, retinoic acid (RA), as the trigger for meiotic initiation in both sexes arose out of studies demonstrating that the entry into meiosis was dependent on expression stimulated by retinoic acid gene 8 (*Stra8*) in both sexes.[16–20] As its name suggests, *Stra8* expression is dependent on RA,[21] and both *Stra8* expression and meiotic entry by female germ cells during embryogenesis can be blocked by RA antagonists.[16–18] These observations were preceded by studies demonstrating the existence of RA receptors (RA receptor (RAR) and retinoid X receptor (RXR)) in embryonic gonads[22,23] and the finding that exogenous RA could induce premature meiosis in fetal ovary cultures.[22,24] The source of RA in vivo has been somewhat contentious, but it is thought to be produced in the neighboring mesonephros via a two-step process that converts retinol into retinaldehyde and then into RA (see Box 1.1). Accordingly, the mesonephros maintains high expression of the genes encoding RA synthetic enzymes, including retinol dehydrogenase 10 (*Rdh10*) and retinaldehyde dehydrogenase 2 (*Raldh2*, also called *Aldh1a2*),[25] along with *Raldh3*/*Aldh1a3*, the latter at lower levels.[26–28] Importantly, however, while the mesonephroi of both male and female embryos can synthesize large quantities of RA,[25] local levels of RA are maintained at 80% lower levels in the developing testis than in the ovaries as a result of high expression of cytochrome

BOX 1.1

RETINOIC ACID SYNTHESIS AND ACTION

Retinoic acid (RA) is a lipid-soluble derivative of vitamin A (retinol) and is essential for many aspects of embryonic development (reviewed by Refs 32,33). RA regulates the expression of genes involved in body patterning and morphogenesis, while disruptions in RA signaling result in a wide range of congenital abnormalities, including skeletal and central nervous defects, cardiac malformation, and craniofacial abnormalities.

Retinol is bound to retinol-binding protein (RPB) for transport through the circulation. Upon reaching its target tissue, defined by the presence of STRA6 receptors on the cell surface, retinol is released from RPB and binds instead to cellular retinol-binding protein (CRBP). Both retinol and CRBP serve as the principal substrate for RA synthesis, through oxidation to retinaldehyde by retinol dehydrogenase 10 (RDH10), but free retinol can also undergo oxidation by more nonspecific alcohol dehydrogenases. This first step of RA synthesis is considered to be rate limiting and is reversible.[34] In light of the higher substrate affinity of RDH10, CRBP provides a substrate specificity mechanism by which specific RDH activity is promoted by the retinol–CRBP complex at the expense of free retinol oxidation through alcohol dehydrogenase.

The second step of RA synthesis is also enhanced through retinaldehyde binding to CRBP, and involves the irreversible oxidation of retinaldehyde to RA by the retinaldehyde dehydrodgenases (RALDHs), of which at least three have been described (RALDH1, RALDH2, and RALDH3; also known as ALDH1A1–3, respectively), encoded in the mouse by the genes *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, respectively. Each of these is expressed in unique patterns across developing mouse embryos, and each results in the production and secretion of RA from the cell to act in a paracrine fashion on neighboring cells and/or tissues.[27,35–38]

Uptake of RA into responding cells is achieved through binding to the cellular retinoic acid–binding protein (CRABP), which prevents the nonspecific degradation of RA in the target cell. Alternatively, RA may bind, and be degraded by, the P450 hydroxylase CYP26 enzymes, which include CYP26A1, B1, C1, and D1, and which may act in a partially redundant fashion to hydroxylate RA into less bioactive 4-oxo-RA. CYP26A1 is itself regulated by RA in a feedback regulatory loop.[32,33]

RA is transported into the nucleus, where it binds to one of a class of three retinoic acid receptors (RARα, RARβ, and RARγ), which are transcription factors of the retinoid receptor family. The retinoid X receptors (RXRα, RXRβ, and RXRγ) also belong to this group and, upon ligand binding, they dimerize with RAR. The RXR–RAR heterodimeric transcription factor, in association with co-activators and histone acetyl-transferase, then associates with RA response elements (RAREs) upstream of transcriptional targets across the genome, thereby initiating gene transcription.

P450 26B1(*Cyp26b1*), a gene that encodes a P450 enzyme that is responsible for degradation of RA[18,29–31] (see Box 1.1), specifically within the supporting somatic cells (Sertoli cells) of the male gonad. Thus, in females, meiosis is brought about by the expression of *Stra8* in PGCs, which is in turn stimulated by the production of RA, presumably in the mesonephros (Figure 1.3(A) and discussed further here). In males, on the other hand, fetal RA production by the mesonephros is counteracted by the expression of *Cyp26b1* by the developing testis, which acts to degrade RA and lower local RA concentrations (Figure 1.3(A)). Expression of *Cyp26b1* begins at 11.5 dpc in males, concurrent with the onset of *Raldh2* expression in the mesonephros of both sexes.[25] (See Refs 27,32–38.)

In male mice, RA production resumes postnatally, at around 3 days postpartum, as the PGC population (now known as *gonocytes*) differentiates into A1 spermatogonia. In both prepubertal and adult testis, *Raldh2/Aldh1a2* expression predominates in the Sertoli cell, although *Raldh1/Aldh1a1* is also expressed to appreciable levels.[39,40] Importantly, *Raldh2/Aldh1a2* is also expressed in premeiotic germ cells of the mouse testis, as well as in pachytene spermatocytes,[40] indicating perhaps some potential for RA synthesis by germ cells themselves. Moreover, studies exploring the relative contributions of different retinoid receptors have indicated that RA may act directly on the Sertoli cells through RARα/RXRβ receptors, as well as through RARγ receptors on spermatogonia[39,41] (Figure 1.3(B)). Thus, it is likely that RA may act in both autocrine and paracrine fashion to facilitate meiotic entry in males. This possibility is supported by recent studies involving cell-specific ablation of all three *Raldh/Aldh1a* genes.[40] Loss of all three *Raldh* genes specifically from Sertoli cells results in a failure of spermatogonial differentiation from A-type to A_1-type, which represents an irreversible first step toward meiotic initiation.[40] This effect is mediated specifically via the RARα receptor on Sertoli cells, indicating an important autocrine effect of RA in this context. Conversely, RA produced by preleptotene spermatocytes (those that are just about to enter prophase I of meiosis) may act in a cell-autonomous fashion to induce meiotic entry

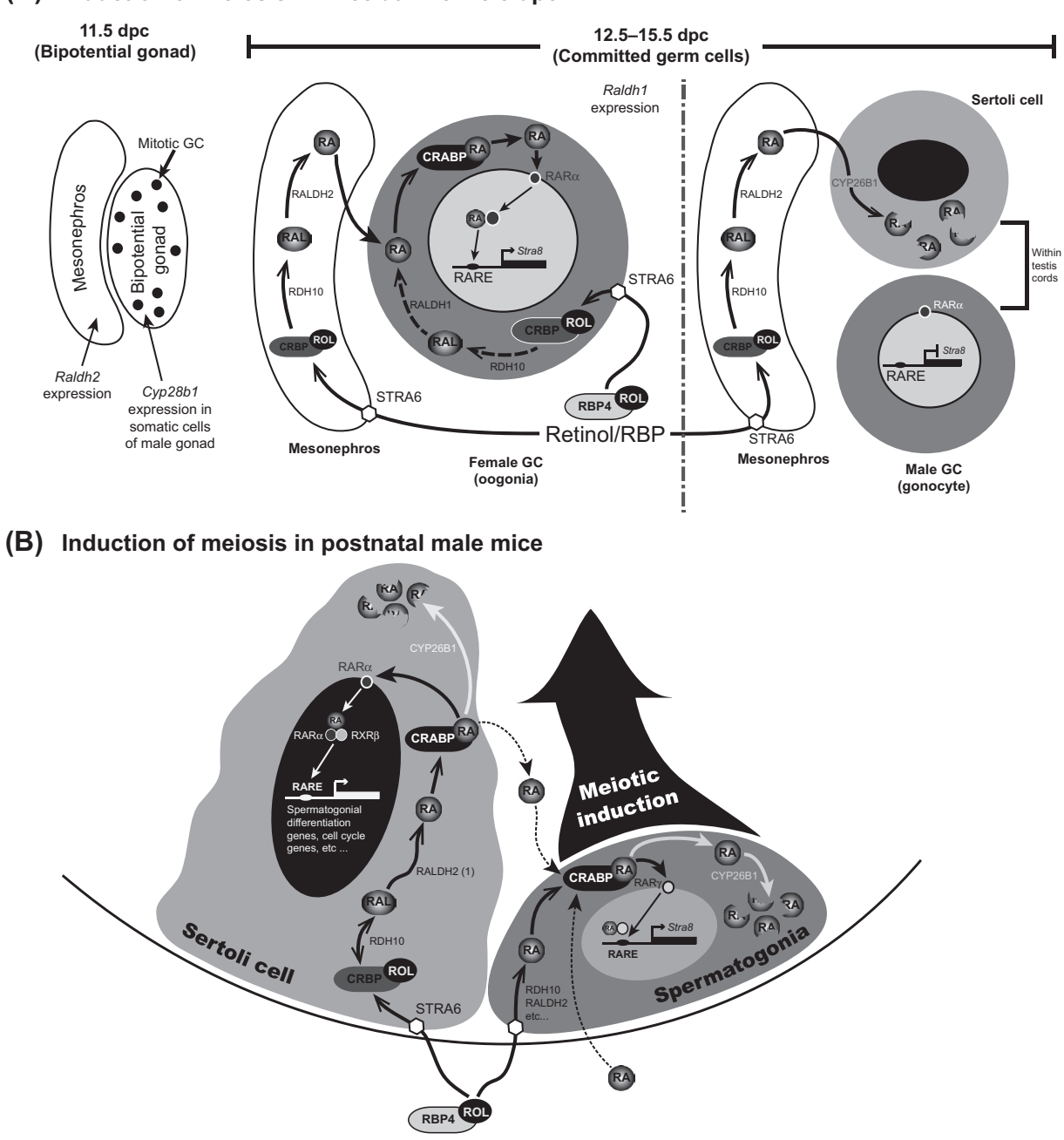

FIGURE 1.3 Induction of meiosis in (A) female and (B) male mice. (A) Females initiate meiosis during embryonic development, starting at around 12.5 dpc. Retinol and retinol-binding protein (RBP) are transported to the mesonephros and taken up via STRA6 receptors. Retinoic acid (RA) is produced through sequential steps, through retinaldehyde, and the RA is then released into the adjoining gonad. Alternatively, RA may be produced directly in germ cells, although this is somewhat contentious at the current time. Once in the oocyte, RA is taken up into the nucleus by binding to RARα, and the complex binds to retinoic acid response elements (RAREs) upstream of RA-responsive genes. In the cell, retinol and its metabolites are bound to appropriate binding proteins (e.g., CRBP and CRABP). In males, RA is degraded in the Sertoli cells of the developing testis cords by CYP26B1, preventing its activity on adjoining male germ cells. (B) In postnatal male mice, retinol is transported to Sertoli cells in which resides all of the appropriate machinery for RA synthesis. CYP26B1 is downregulated in the Sertoli cell, resulting in elevated RA levels. This RA is directed toward adjoining spermatogonia, and can induce expression of RA response genes through RA–RARγ uptake into the nucleus. Once again, CYP26B1-mediated degradation of RA is prevented by downregulating its expression in male germ cells. See the accompanying text for further details.

via RARγ-mediated induction of *Stra8* expression[17,40,41] (Figure 1.3(B)). Importantly, however, the requirement for autocrine signaling of RA within the Sertoli cell to induce initial spermatogonial differentiation appears necessary only for the first wave of spermatogenic initiation, and is dependent on RARα, while RARγ is not required,[41] presumably because it resides within the germ cells. Conversely, Sertoli cell RA signaling through RARα does not appear to be required for subsequent waves, the induction of RA being now relegated presumably to the germ cells.[30] All of this information notwithstanding, it seems that the question of where RA is synthesized in the postnatal testis is not yet fully resolved, and more studies are required to confirm the spermatogonia and spermatocyte populations as sources of autocrine RA.

This potential role of germ cells in inducing meiotic initiation helps to clarify an important controversy regarding the role of RA in meiotic entry. Kumar et al.[42] utilized female mice bearing mutations in the *Raldh2/Aldh1a2* gene, or in both the *Raldh2/Aldh1a2* and *Raldh3/Aldh1a3* genes specifically in the mesonephros during embryogenesis, and showed that *Stra8* expression was still observed in fetal oocytes.[42] They also demonstrated that inhibition of CYP26B1 activity with ketokonazole allows for RA-independent induction of *Stra8* expression in the wild type or in the mutant embryos, but only if the mesonephros remains intact, while RA activity is not detected in wild-type fetal testes following ketoconazole treatment.[42] Taken together, these findings would suggest (1) that RA does not act directly on the gonad to induce meiotic entry during fetal life, and (2) that CYP26B1 acts to inhibit entry into meiosis but not at the level of gonadal RA activity. The authors concluded from these observations that RA was not acting to promote meiotic entry since they could find no evidence of another (gonadal) source of RA that could be responsible for meiotic initiation in females. Furthermore, they concluded that the target of CYP26B1 was a non-RA molecule within the mesonephros.[42] However, these data are in line with those described for the male here, and lead to a model in which RA produced by the developing ovary, and more specifically by the oogonia, is responsible for inducing meiotic progression either in a cell-autonomous manner to promote *Stra8* expression and/or indirectly by acting in a paracrine fashion to induce RA-responsive genes in the mesonephros (Figure 1.3(A)). Thus, ketoconazole inhibition of CYP26B1 could result in the loss of RA signaling specifically in the mesonephros, but with the RA originating from the fetal gonad. In support of this, a recent report suggests that RA can be produced from retinol by PGCs in the fetal ovary, with the RALDH1/ALDH1A1 protein being localized specifically in germ cells from 12.5 to 15.5 dpc[43] (Figure 1.3(A)). Such an observation is supported by those early studies of ectopic germ cell entry into meiosis, which is well clear of any influence of the mesonephros.[14] Taken together, these recent studies lend support to the idea that the germ cells themselves, rather than supporting cells and tissues, are the source of the RA that triggers meiotic progression, but that the effect of RA may require these supporting cells for paracrine interactions that lead to the onset of meiotic events.

Stra8 and the Induction of Meiosis

The major target of RA activity in mouse germ cells is to induce the expression of *Stra8*,[18,25] a gene initially described and characterized in P19 embryonal carcinoma cells.[21,44] In the mouse embryo, *Stra8* expression is observed from 12.5 dpc in females, approximately one day prior to the onset of meiosis. Prior to this time, male and female germ cells are indistinguishable, expressing similar genes such as Deleted in azoospermia-like (*Dazl*; Figure 1.4(A) and (B)). Interestingly, the entry into meiosis is not synchronous, but instead occurs in an anterior-to-posterior wave through the ovary.[45,46] Thus, germ cells in the anterior region of the ovary are exposed to RA, express *Stra8*, and enter meiosis from 12.5 dpc, while those of the posterior end enter somewhat later, with RA activity being evident from 13.5 dpc and *Stra8* expression extending to 16.5 dpc.[8,45] This *Stra8* expression occurs under a backdrop of high RA levels, coincident with downregulation of *Cyp26b1* expression (Figure 1.4(A), (C)–(F)), and leads within hours to the downregulation of pluripotency markers, such as POU-domain class 5 transcription factor 1 (*Pou5f1*; also known as octamer-binding transcription factor 4 (*Oct4*)), and the increased expression of meiotic markers such as Synaptonemal complex protein 3 (*Sycp3*), *Spo11*, and the meiosis-specific RecA homolog, Disrupted meiotic cDNA 1 (*Dmc1*).[9,25,47] *Stra8* expression is not required for proliferative activity of the female germ cells, but appears instead to be essential for the premeiotic S phase and meiotic entry.[16] The evidence that similar mechanisms exist in humans is provided by the observation that RA can induce *STRA8* expression in human fetal gonad explant cultures.[48,49]

In males, as in females, RA leads to induction of *Stra8*, but this occurs postnatally and usually around the time of puberty in most mammals. In mice, however, *Stra8* induction occurs co-incident with downregulation of *Cyp26b1* during early postnatal life, from around day 8 postpartum (pp), immediately prior to the onset of meiotic prophase I (Figure 1.4(A), (D), and (E)).[50] Loss of *Stra8* in males appears to result in a failure to transition from preleptotene stages into prophase I, but this appears to be a background-specific phenomenon, since the failure to enter meiosis

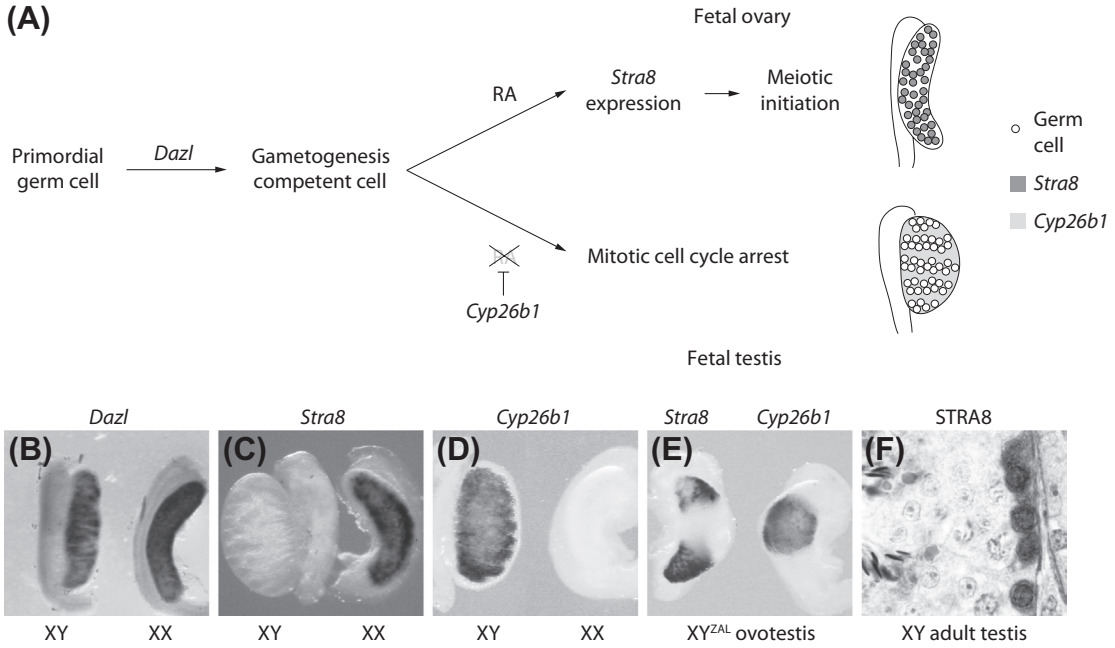

FIGURE 1.4 Regulation of meiotic initiation in mice. (A) Ovarian germ cells initiate meiosis during embryogenesis, whereas testicular germ cells undergo G0/G1 mitotic cell cycle arrest and do not initiate meiosis until after birth. Primordial germ cells in the fetal gonad express *Dazl*, thus acquiring competence for gametogenesis and gaining the ability to interpret RA as a meiosis-inducing signal. In the fetal ovary, signaling by retinoic acid (RA) induces *Stra8* expression and thereby meiotic initiation in germ cells. In the fetal testis, cytochrome p450 (*Cyp26b1*), an RA-metabolizing enzyme, is expressed in somatic cells surrounding the germ cells and prevents premature *Stra8* expression. (B–D) Whole-mount in situ hybridization on fetal testes and ovaries (B) at embryonic day 13.5 (E13.5) for *Dazl*; and at E14.5 for (C) *Stra8* and (D) *Cyp26b1*. (E) In E14.5 XY^{ZAL} ovotestes, *Stra8* is expressed in the polar regions exhibiting ovarian histology, and *Cyp26b1* is expressed in the central region exhibiting testicular histology. XY^{ZAL} embryos carrying a Zalende-derived Y chromosome on a C57BL/6 genetic background are often partially sex-reversed and have ovotestes. (F) Immunohistochemistry for STRA8 in adult testis. STRA8 is expressed in preleptotene germ cells in stage VII/VIII tubules. *Source: Figure kindly provided by Dr David Page and Shirleen Soh, Whitehead Institute, MIT, with additional images provided by Yanfeng Lin (panel B) and Tsutomu Endo (panel F). (A, C, D, E) Adapted from Refs 16,18,45,492,493.*

is strain specific.[16,17,51] In one *Stra8* mutant mouse line, meiotic entry was not impeded by the loss of *Stra8*, but instead meiotic progression was disrupted as a result of premature chromosome condensation.[51] As a result, Hogarth et al. concluded that genes other than *Stra8* must also be required to promote meiotic entry, and they went on to identify three potential candidates, at least one of which was RA responsive. Indeed, the RA-responsive gene, Establishment of cohesion 1 homolog 2 (*Esco2*), is expressed in embryonic ovaries at around 14.5 dpc, and in postnatal testis at day 10 pp, the protein appears to reside within the nucleus and is an essential component of the sister chromatid cohesion complex.[50,52] The roles of *Esco2* as well as of other genes whose profiles correlate with *Stra8* expression, such as *Setdb2* and *Uba6*,[50] remain unclear at the current time.

Recent studies have identified the mammalian Doublesex-related transcription factor 1 (DMRT1) as a transcriptional repressor of *Stra8* in both male and female germ cells.[53,54,55] However, it appears that the effect of DMRT1 on meiotic initiation is cell context specific and perhaps relies on differential posttranslational modification of the protein, resulting in sexually dimorphic regulation of meiotic entry. In adult males, expression of *Dmrt1* prevents the transition from spermatogonial to spermatocyte stages by limiting RA-induced transcription of *Stra8*, and at the same time promotes spermatogonial development by induction of genes required for differentiation from A- to B-type spermatogonia, including the Spermatogenesis and oogenesis-specific helix–loop–helix 1 transcription factor (*Sohlh1*).[54] DMRT1 was shown to bind to the *Stra8* promoter close to the RA response elements, presumably preventing binding of RA and thus limiting meiotic entry.[54] Interestingly, DMRT1 also alters expression of *Cyp26b1* and some of RA's synthetic enzymes, suggesting a direct action of DMRT1 on RA production and stability.[54] Alternatively, given recent evidence that pachytene spermatocytes can synthesize RA,[40] the reduced expression of these RA enzymes in the absence of DMRT1 may result from a loss of cells that are themselves a major source of RA. In either case, it appears that DMRT1 may modulate RA responsiveness

by spermatogonia, thereby providing a modulating influence for meiotic entry in the premeiotic cell population. Taken together, these new observations present a mechanism by which meiotic entry is controlled by the exquisite interplay between opposing pathways to both limit and regulate how and when cells can initiate prophase I.

Unlike the situation in postnatal males, DMRT1 does not appear to play a role in preventing meiotic entry in fetal mouse testes.[55] *Dmrt1* expression arises prior to sex determination in the bipotential gonad at 11.5 dpc,[53,56] but its expression in the ovary is transient, disappearing from somatic cells by 13.5 dpc and then from germ cells by 15.5 dpc.[53,56] Despite this transient expression, loss of *Dmrt1* in *Dmrt*$^{-/-}$ female embryos results in significantly reduced expression of *Stra8* and a consequent failure to form the full complement of primordial follicles.[53] The level of expression of *Stra8* is sufficient to allow some cells to enter meiosis, albeit aberrantly, but these cells subsequently fail to proceed normally through prophase I. This is exactly the opposite situation to that seen in postnatal male germ cells, in which DMRT1 is required to suppress meiotic entry through downregulation of *Stra8* expression.[54]

Sry Induction of the Male Germ Cell Program during Fetal Development

While the destruction of RA by CYP26B1 is a major mechanism by which male germ cells avoid meiotic entry, a recent study has suggested that secretions from cells other than the germ cells may also function to restrict meiotic entry by male germ cells in utero. This was demonstrated by the observation that XY germ cells in cultured mouse fetal testes can occasionally enter meiosis when secretory activity is prevented by the compound Brefeldin A.[57] One such secretory protein appears to be fibroblast growth factor 9 (FGF9), which is essential for male germ cell survival during fetal life, and deletion of which is known to result in male-to-female sex reversal.[58,59] *Fgf9* expression arises in the somatic cells of the bipotential gonad prior to gonadal sex determination, but by 11.5 dpc becomes restricted to the developing testis,[60,61] having been suppressed in females by the female factor gene, wingless-related MMTV integration site-4 (*Wnt4*) (reviewed by Ref. 62). FGF9 treatment of cultured mouse gonads restricts meiotic entry, resulting in reduced *Stra8*, *Dmc1*, and *Sycp3* expression,[61,63] but at the same time it promotes the expression of pluripotency factors such as *Oct4* and Sex determining region Y (Sry)-box 2 (*Sox2*), along with markers of male fate, including Nanos homolog 2 (*Nanos2*) and DNA methyltransferase 3-like (*Dnmt3L*).[61,63] The action of FGF9 occurs directly on germ cells, most likely via the FGF receptor FGFR2, which is present on the surface of germ cells at 12.5 dpc. It has been postulated that the action of FGF9 on germ cells results in upregulation of the RNA-binding protein NANOS2 in primordial germ cells of both sexes and in premeiotic spermatogonia, leading to suppression of meiotic entry.[63] Studies using in vitro culture of fetal tissues indicate that FGF9 most likely acts by reducing the responsiveness of germ cells to RA, possibly via *Nanos2* regulation, reducing the ability of RA to induce *Stra8* expression,[64] while at the same time suppressing expression of *Wnt4*.[65] Thus, loss of *Fgf9* induces upregulation of *Wnt4*, which induces germ cells to become feminized, while loss of *Wnt4*, in turn, induces a male fate via upregulation of *Sox9* (reviewed by Ref. 66). *Sox9* is a direct target of the sex determination gene *Sry*, whose expression in males initiates a feedforward loop between *Sox9* and *Fgf9* (Figure 1.5), leading to upregulation of the latter.[65] Taken together, these observations suggest that suppression of meiotic entry in male gonads during embryogenesis is brought about by SRY-induced *Sox9* expression that, in turn, results in the expression of *Fgf9* (Figure 1.5(A)). The action of FGF9 is to suppress *Wnt4* expression and to impair the ability of male germ cells to respond to RA, through mechanisms that remain largely undefined. In addition, expression of *Cyp26b1* is enhanced both by SOX9 and by the transcription factor steroidogenic factor 1 (SF1) to promote RA degradation (Figure 1.5(A)).[67]

In the absence of SRY in females, germ cell sex determination is marked by increased expression of *Wnt4*, whose expression is regulated by R-spondin1 (RSPO1), leading to suppression of *Sox9* and *Fgf9* expression.[62]

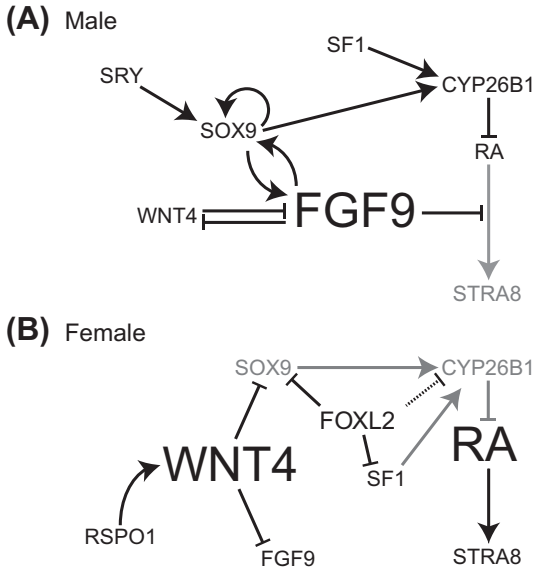

FIGURE 1.5 Summary of key sex determination signals that inhibit retinoic acid (RA) induction in male germ cells (A), and those that induce RA in female germ cells during embryonic development (B). See the main text for further details.

At the same time, elevated expression of forkhead transcription factor 2 (*Foxl2*) acts to suppress production of CYP26B1 through several mechanisms: by further suppressing *Sox9* expression, by repressing *Sf1* expression,[67] and possibly also by other mechanisms. Thus, in females, the drivers of sex determination consist of WNT4 and FOXL2, which help to promote meiotic entry via suppression of *Cyp26b1*, thereby stabilizing RA levels in the gonad (Figure 1.5(B)). Whether WNT4 and FOXL2, or indeed any other factors, have a direct effect on RA metabolism and action is uncertain at the current time.

Early Indications for a Role for Small RNAs in Meiotic Entry

Recent studies have indicated a role for small RNA species in meiotic initiation. Members of the microRNA family *Mirlet7*, for example, are expressed in premeiotic and meiotic germ cells in response to RA signaling.[68] The induction of *Mirlet7* expression by RA signaling has been shown to occur via repression of the RNA-binding protein LIN28.[68] Furthermore, in mice lacking the Argonaute 4 (*Ago4*) gene, which encodes a member of the AGO family of proteins that are defining components of the small RNA–processing machinery, early postnatal spermatogonia enter meiosis approximately 4–6 days early.[69] This precocious entry into meiosis is associated with early upregulation of genes such as *Stra8*, *Esco2*, and *Sycp3*, and also altered expression of genes encoding the RA synthetic enzymes and *Cyp26b1*, suggesting that small RNAs that associate with, and function alongside, AGO4 may modulate meiotic entry through feedback mechanisms that regulate RA metabolism. These studies are at a very early stage, but they raise the possibility that meiotic induction and RA metabolism in germ cells are intricately linked to small RNA biogenesis and action.

PROPHASE I

Overview of Prophase I

Events during the first meiotic stage (meiosis I) are designed expressly to ensure that homologous chromosomes (one maternal and one paternal in origin) segregate equally at the first meiotic division (Figure 1.1). By contrast, during the second meiotic division, as in mitosis, it is the sister chromatids that must segregate equally. For meiosis II and mitosis, this is relatively easy, since the sister chromatids are already tethered together via the process of cohesion (discussed further in this chapter). It stands to reason, therefore, that the homologous chromosomes of meiosis I must first find a similar pairing mechanism that will enable them to segregate in a similarly efficient manner. Thus, the necessity of homologous chromosome pairing adds a level of complexity to meiosis I that is unique and defining for this stage. These events all take place during prophase I, and pairing is ensured by two distinct mechanisms, synapsis and recombination, the former through protein–protein interactions and the latter through DNA–DNA interactions. Both events require that homologous chromosomes must first locate each other, recognize their homologs, and then interact physically, and both are specifically designed to allow the tethering of homologs until the first meiotic division, much as sister chromatids are physically connected through cohesion.

As a result of the added complexity, meiotic prophase I is extended compared with its mitotic counterpart, and can take several days to complete in mammalian species. Separated into five distinct substages (Figures 1.6 and 1.7(A)–(E)) based on the cytological appearance of the chromosomes, prophase I is the most complex and long-lived stage of meiosis. Figure 1.6 summarizes the events of prophase I in temporal order from left to right, including the synapsis events (described in the upper portion of the figure) and recombination events (described in the lower portion of the figure). The status of the sister chromatids and homologous chromosomes is depicted in cartoon form in the middle of Figure 1.6.

The first substage of prophase I is known as leptonema (from the Greek *leptos*, meaning "thin"). It is the stage immediately following premeiotic DNA replication where the chromosomes are still fairly long, decondensed, and threadlike. The main event during leptonema involves the initial assembly of a proteinaceous structure, termed the synaptonemal complex, which begins to form a scaffold along each homologous chromosome between the sister chromatids. These scaffolds are termed axial elements, and they first assemble as short stretches along each homologous chromosome, the stretches coalescing to become longer filaments until they extend down the entire length of the chromosome in late leptonema and into the zygotene stage. Concurrent with synaptonemal complex assembly, meiotic cohesins are also assembled onto the homologous chromosomes, and subsequently disassembled, a process that is discussed in detail in this chapter.

During the second substage of prophase I, termed zygonema (from the Greek *zygon*, meaning "adjoining"), homologous partner chromosomes begin to pair, and then to synapse, the latter resulting in the physical tethering between homologs. Simultaneously, the DNA begins to condense, making the chromosomes more visible cytologically. This process of synapsis requires that the axial elements along each

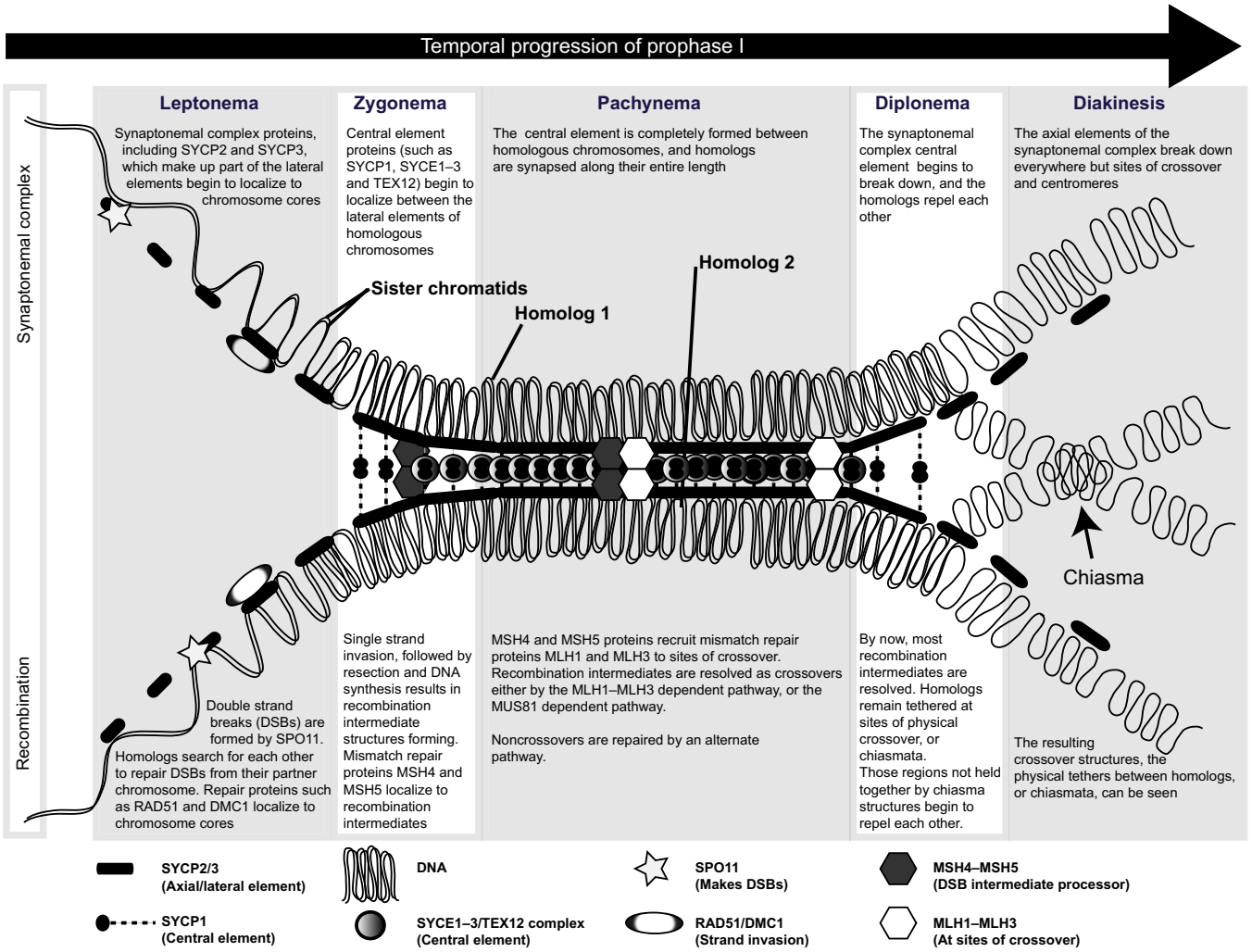

FIGURE 1.6 Dynamics of recombination and synapsis in prophase I. Events occurring during the five substages of prophase I (leptonema, zygonema, pachynema, diplonema, and diakinesis) are depicted schematically, according to their involvement in either synapsis events (along the top) or recombination events (along the bottom).

homolog are stabilized and joined together in a zippering process that involves multiple protein complexes, collectively termed the central element. Once the homologs have been joined together by the central element, they are said to have synapsed, and, once this occurs, the axial elements are reclassified as lateral elements. Upon complete synapsis along the entire length of the homologous axes, the cell enters the third substage of prophase I, known as pachynema (after the Greek *pachy*, meaning "thick", given that the chromosomes are now fully condensed and have a short, fat cytological appearance). Pachynema is the longest of the substages and can last up to 10 days in some rodent species, and even longer in humans. Once pachynema is complete, the central element of the synaptonemal complex begins to break down, and the chromosomes begin to repel each other. This fourth substage is known as diplonema (from the Greek *diplo*, meaning "double", given that two homologs become clearly visible again). Despite the central element breaking down and the synaptonemal complex disassembling, the homologs remain joined at the physical sites of meiotic COs, called chiasmata. These COs are generated by the process of repairing meiotic double-strand breaks (DSBs), discussed further in this chapter, in a process that is initiated in preleptonema and leptonema and that progresses concurrently with synaptonemal complex assembly. The fifth and final substage of prophase I, diakinesis, is named for the Greek *dia* (meaning "through") and *kinesis* (meaning "movement"), so in essence it means "moving through", indicating the final stage before proceeding to the next phase of meiosis, metaphase I. At this point, the homologs are connected only by the remnants of the synaptonemal complex found at the centromeres and at the CO sites.

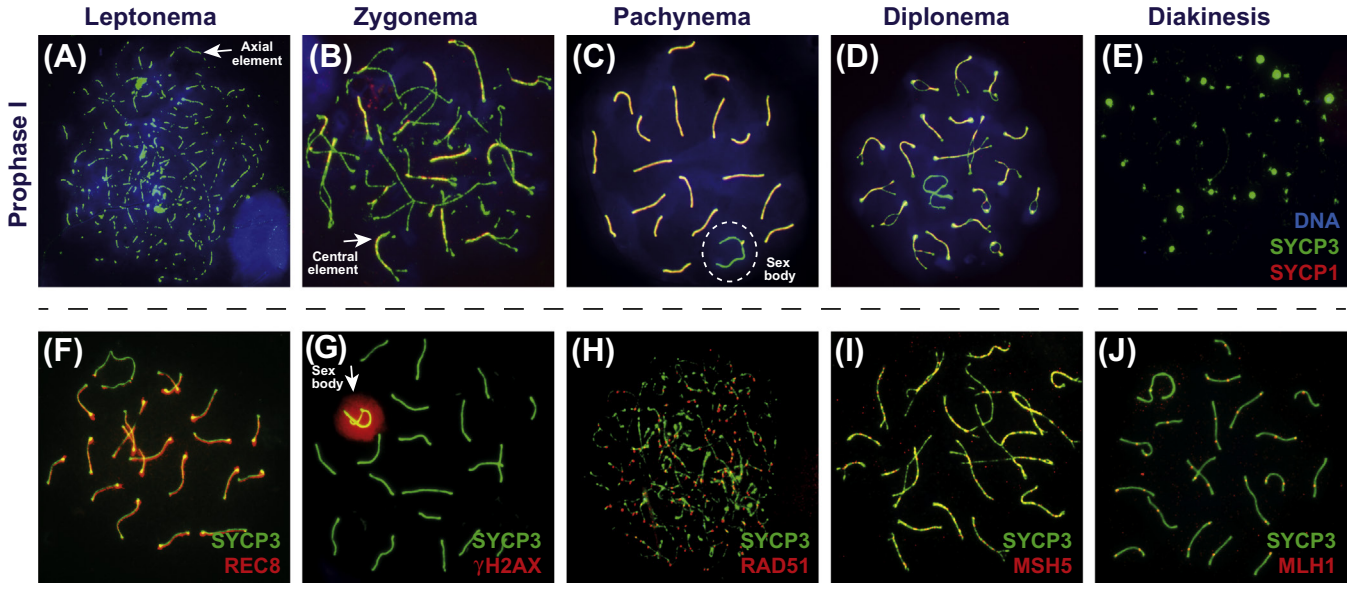

FIGURE 1.7 Immunofluorescent analysis of meiotic prophase I events in mouse spermatocytes. This figure is reproduced in color in the color plate section. (A–E) Synaptic events in the five substages of prophase I, visualized by staining with antibodies against axial–lateral element protein SYCP3 (green) and central element protein SYCP1 (red), where the overlay between the two appears in yellow. (F–J) Immunolocalization of SYCP3 (green) with key meiotic markers during prophase I; (F) REC8, (G) λH2AX, (H) RAD51, (I) MSH5, and (J) MLH1 all in red, and SYCP3 in green.

Chromosome Dynamics and Pairing in Early Prophase I

Chromosome Movements, Telomere Clustering, and Telomere Bouquet Formation

One of the fascinating aspects of meiotic prophase I is the delicate, yet dramatic, orchestrated "dance" that occurs between homologous chromosomes. The reorganization of the nuclear contents, and their situation with respect to the nuclear envelope, are, quite understandably, critical to the success of homolog alignment and, as a consequence, are essential for prophase I progression. It is notable that although there exists a large body of data concerning the molecular events that occur following synapsis of chromosomes, far less is known about how homologs recognize and pair with one another. Almost certainly, numerous mechanisms exist to promote homolog recognition and pairing, and these may be utilized to differing degrees in different organisms. For example, in many organisms, while pairing may occur at numerous sites along chromosomes, there are often defined locations where initial pairing interactions take place. The so-called pairing centers of *C. elegans* are a strong example of this.[70] The congregation of pairing centers coincides with the generation of highly conserved rapid chromosome movements, which have been demonstrated for many species, including mammals and both budding and fission yeast.[71,72] Driven by the Sad1p, UNC-84 (SUN)–Klarsicht, ANC1, syne homology (KASH) family of nuclear membrane–bound proteins, rapid chromosome movements are propagated by telomere connections to cytoplasmic motors that drive oscillatory movements.

Another important feature of chromosome interactions during early prophase I is the "telomere bouquet". The bouquet describes the clustering of telomeres at one location across the nuclear envelope, and is noted in many meiotic species.[73,74] However, the relative importance of rapid chromosome movements and bouquet formation appears to be species specific, since, for example, in *Sordaria macrospora*, the bouquet stage follows homolog pairing[75] and also follows early synapsis in female mice,[76] while in *Saccharomyces cerevisiae*, rapid chromosome movements appear to be more critical than bouquet formation for pairing.[77]

The oscillatory rapid chromosome movements of the fission yeast, *S. pombe*, are particularly noteworthy due to their long-lived activity and their dramatic motion through the nuclear space. During this so-called horsetail motion,[78] the telomeres gather in a tight bouquet adjacent to the spindle pole body (the microtubule organizing center that is itself outside the nuclear membrane on the cytoplasmic side) early in prophase I. The spindle pole body then proceeds to drag the entire nucleus backward and forward, powered by a meiosis-specific dynein motor while meiotic recombination is taking place.[79] Mutants in which telomeres coalesce normally but that are incapable of spindle pole body–driven nuclear movements show more severely impaired

meiotic recombination than mutants with impaired bouquet formation, indicating that the horsetail movements of the nucleus are more critical for recombination events in *S. pombe*.[73] In contrast, mutants that exhibit altered bouquet formation have more defective asci (spore-bearing cells), suggesting that the bouquet may play additional roles in later or additional meiotic events.

Telomere clustering also plays a prominent role in meiotic prophase I in budding yeast (*C. elegans*) and in mammals (reviewed in Refs 80,81,82). However, the relative importance of rapid chromosome movements and bouquet conformations for homolog pairing and synapsis in mammals is relatively less well understood. In early prophase I in mouse meiocytes, rapid chromosome movements are readily observed and can be visualized in real time.[83] In addition, telomeres attach to the nuclear envelope via a conical thickening of the axial element.[81,82] These telomeres coalesce, as they do in other organisms, to form a defined bouquet structure. Moreover, in male mice, the bouquet is short-lived and often hard to find, but occurs concomitantly with homolog alignment and pairing, calling into question whether pairing is dependent on these telomere events in mammals. Loss of synaptonemal complex protein 3 (SYCP3) does not affect bouquet formation, despite the loss of axial elements,[84] whereas loss of the cohesion component (structural maintenance of chromosomes 1 beta (SMC1β)) results in failed nuclear envelope attachment.[85] Telomeric repeats also appear important for the clustering of telomeres in mouse, yeast, and worm (reviewed in Ref. 82).

Involvement of SUN–KASH Proteins in Telomere Tethering to the Nuclear Envelope

At the molecular level, the pathways that regulate telomere movements and bouquet formation are only just being elucidated, most of the prominent players having been identified in fission yeast. In *S. pombe*, Bqt1 and Bqt2 proteins connect telomeres to the spindle pole body via their interactions with Rap1, and, together, these three proteins form a bridge from the telomere to Sad1, a spindle pole body protein associated with the inner nuclear membrane.[86,87] Rap1, in turn, requires Taz1 for its association with the telomere in *S. pombe*.[88] Sad1 is the founding member of the SUN domain proteins, whose orthologs are present across the spectrum of meiotic species.[89] For example, in budding yeast, the telomere-associated protein Ndj1 is critical for telomere movements and bouquet formation,[82,90,91] and its function is dependent on interactions with the SUN domain protein, Mps3, present on the inner nuclear membrane.[91,92]

SUN proteins function together with KASH domain proteins, located on the outer nuclear membrane, forming a bridge that links telomeric DNA ends with specific elements of the cytoskeleton.[93] KASH domain proteins include Kms1 in *S. pombe* and ZYG12 in *C. elegans*, and they have also been reported for mammals (reviewed by Refs 89,94,95). In budding yeast, in contrast, Csm4 is required for telomere clustering and for homologous recombination[91,96,97] and, while not strictly a KASH domain-containing protein, appears to interact with Mps3 and Ndj1.[89]

In mammals, at least six SUN proteins have been described, only some of which have been associated with a specific KASH protein (reviewed by Refs 94,95). The two major SUN proteins involved in mammalian meiosis are SUN1 and SUN2, both of which utilize a common meiosis-specific KASH protein, KASH5, for tethering to cytoplasmic microtubules.[95,98,99] Localization of both SUN1 and KASH5 is restricted to meiotic prophase I, and more specifically to the telomeric regions of chromosomes during leptotene of prophase I onward.[98,100] Loss of *Sun1* in mice results in loss of telomere attachment to the nuclear envelope and synapsis failure during prophase I, resulting in male and female sterility.[100] Similarly, mutation of the *C. elegans* ortholog of *Sun1*, Matefin, disrupts its interaction with the KASH protein, ZYG12, and prevents synapsis and homologous recombination.[101]

What is clear from all these comparative studies of chromosome dynamics is that chromosomal connection to the cytoskeletal machinery, via the nuclear envelope, is a conserved feature of early prophase I progression and is essential for initial chromosome interactions and/or recombination. Conserved protein families mediate these interactions, including proteins of the SUN and KASH family (Figure 1.8). Most commonly, the attachments at the telomere may involve meiosis-specific proteins, such as Ndj1 in budding yeast and Bqt1 and Bqt2 in fission yeast, although no such linker protein has yet been identified in mammals. Rap1, which is essential for such interactions in fission yeast, appears to play no role in telomere attachment during meiosis in mice.[102] The SUN and KASH components involved in telomere clustering and homolog pairing in the plant kingdom remain to be elucidated, but are almost certainly conserved like their animal kingdom counterparts.

Pairing Centers and Noncanonical Mechanisms to Induce Homolog Pairing

It is interesting to note that in organisms that do not display a defined bouquet, nuclear envelope interactions remain critical for prophase I progression. *Caenorhabditis elegans* is an example of this in which pairing is mediated by pairing centers,[70] which themselves associate with the nuclear envelope during early stages of prophase I.[103] Pairing centers on each chromosome are bound by a single member of a family of zinc finger proteins, HIM8, and the ZIM proteins, ZIM1, ZIM2, and ZIM3.[103] These proteins mediate

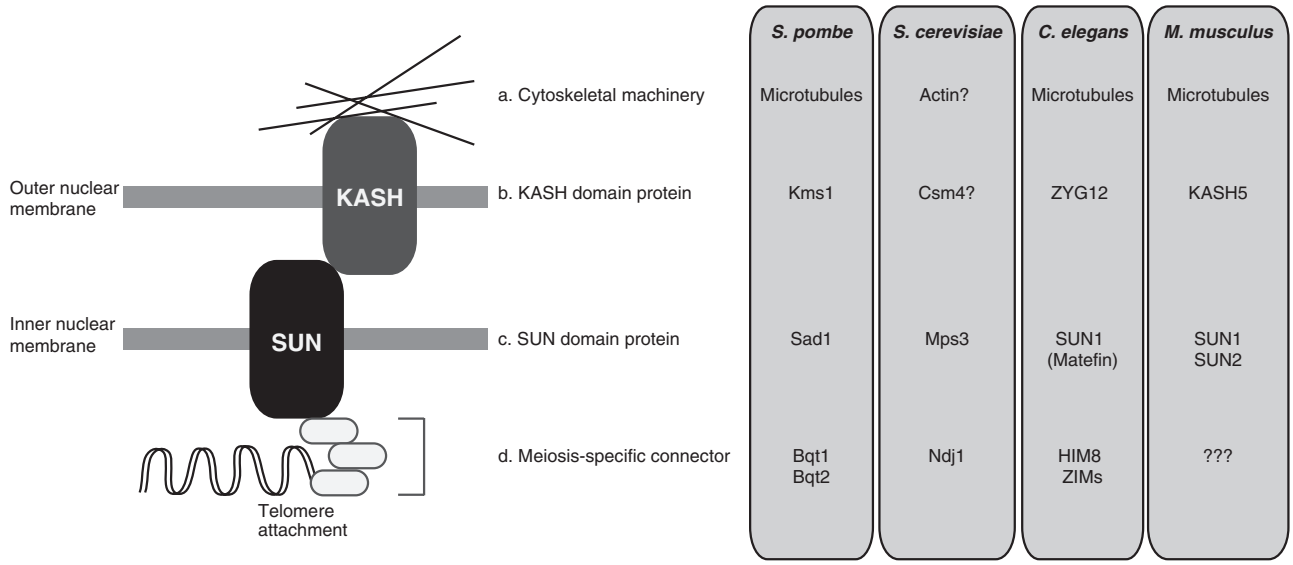

FIGURE 1.8 Chromosome movement and association with the nuclear envelope are coordinated through the highly conserved SUN–KASH family of nuclear membrane proteins. The left panel represents a simplified cartoon of SUN–KASH interactions with meiotic chromosomes and with the cytoskeletal machinery. On the right, known mediators of these events are shown for fission yeast, budding yeast, worms, and mice.

binding of the pairing center to the nuclear envelope in a bouquet-like fashion. Thus, while no specific bouquet is formed, the nucleus of early prophase I meiocytes in *C. elegans* displays a distinctly polarized appearance, and the chromosomes are clearly asymmetrically distributed.[104] This nuclear association of chromosomes is dependent on the SUN protein Matefin (SUN1) and the KASH protein ZYG12.[101,105] Thus, nuclear attachment is mediated at pairing centers rather than at telomeres, but is also mediated through SUN–KASH interactions with the ZIM proteins.

A recent report from studies in *S. pombe* indicates that a novel RNA-induced homolog-pairing process may exist in this organism.[106] Ding et al. demonstrate that the *SME2* locus is among the first to pair during the horsetail stage. This locus encodes a meiosis-specific noncoding RNA that appears to mediate homolog recognition. In *S. pombe*, meiotic initiation is regulated by Mei2, an RNA-binding protein. Mei2 forms a distinct focus at the *SME2* locus, and it sequesters Mmi1, another RNA-binding protein that is required for elimination of meiosis-specific mRNA transcripts in mitotic cells. Thus, the Mei2-induced removal of Mmi1 allows meiotic progression. At the same time, the binding of Mei2 and Mmi1 to *SME2* transcripts is sufficient to induce pairing at this locus, as well as between ectopic loci on nonhomologous chromosomes. It appears, however, that while *Sme2* transcripts can act as *cis*-acting pairing factors, there may be other, similar noncoding RNA loci that play similar roles in pairing and meiotic initiation across the *S. pombe* genome.

The Synaptonemal Complex and Synapsis

The synaptonemal complex was first visualized by Moses and Fawcett in 1956.[107,108] As a complete tripartite protein structure, it consists of two lateral elements that align each homolog and the central element. In addition, transverse filaments have one end anchored in the lateral element and the other in the central element, and function to "zipper" the two homologs together during early prophase I, in the process known collectively as synapsis (Figure 1.6). In the mouse, a significant amount of chromosome pairing occurs premeiotically, prior to the initiation of the DSBs at leptonema, and is dependent on the SPO11 and SUN1 proteins.[109]

Upon entry into meiosis, in the leptotene stage, the synaptonemal complex begins to form short stretches of axial element along the sister chromatids of each homologous chromosome, with the chromatin extending perpendicularly out from the development axial element to form loops (Figure 1.7(A)). The two major components of the axial elements are SYCP2 and SYCP3, first identified in the rat but later identified in many other mammalian species.[110,111,112,113] SYCP3 consists of two proteins in mice and rats (30 and 33 kDa in size) that are transcribed from the same gene, with the shorter isoform very highly conserved among vertebrate species,[114] whereas SYCP2 is a much larger protein at 173 kDa.[113] SYCP2 and SYCP3 form heterodimers that are dependent upon the conserved coiled-coil domain in SYCP2. Both proteins begin to localize to chromosomes to form axial elements during leptonema.[115,116] Mutations in either *Sycp2* or *Sycp3* in the mouse result in a

similar phenotype, namely, the absence of functional axial elements and proper synapsis, along with impaired specificity of chromatin loop attachment, resulting in male sterility and female subfertility.[84,115,117,118] Intriguingly, mutant SYCP2 protein still localizes to the axial element in certain *Sycp2* mutants, but it cannot recruit SYCP3, demonstrating that functional SYCP2 is required for SYCP3 localization.[115] In addition, SYCP2, but not SYCP3, is known to be a physical link between the transverse filament and the axial element.[119,120]

During zygonema, homologous chromosomes are joined together in a "zipper"-like fashion by the transverse filament and central element (Figure 1.7(B)). This tripartite structure is highly evolutionarily conserved among species, from yeast to worm, mouse, and human. The C-terminal of SYCP1 is known to embed within the lateral element of the synaptonemal complex, while the N-terminal region is known to associate with the central element (Figure 1.6, pachynema). Additionally, the N-terminal of SYCP1 interacts strongly with itself, suggesting that SYCP1 subunits lie head-to-head within the space between the lateral elements of homologous chromosomes, and physically associate with the central element, effectively joining the two elements together.[119] More recent evidence shows that upon its localization, SYCP1 recruits other proteins to the central element, such as SYCE1, SYCE2, SYCE3, and TEX12.[121,122,123] SYCE1 and SYCE2 bind to one another and to SYCP1, while TEX12 forms a complex with SYCE2, thus only forming an indirect interaction with SYCP1.[122,124] Localization patterns of SYCP1, SYCE1, and SYCE3 are similar, forming a continuous staining pattern along the synaptonemal complexes of synapsed chromosomes,[121,123] whereas SYCE2 and TEX12 display a more punctate staining pattern and do not necessarily co-localize with SYCP1.[122,123] The complex formed by SYCE2 and TEX12 is thought to have an architectural purpose within the central element of the synaptonemal complex, as it consists of a hetero-octamer made up of one SYCE2 tetramer and two TEX12 dimers bound together.[124] It is postulated that this complex, within the confines of a "zipper" analogy for the synaptonemal complex, would be the slider that pulls the SYCP1–SYCE1–SYCE3 "teeth" together to zip up the synaptonemal complex. Other protein components are thought to be members of the central element scaffold, such as FKBP6, although very little is known about their role in synaptonemal complex assembly.[125] Recent studies suggest that FKBP6 may be a player within the RNA interference (RNAi) pathway during meiotic prophase I, with a role specifically in the biogenesis of P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) in premeiotic mouse testes, and in suppressing LINE1 retrotransposon integration into, and thus disruption of, the genome.[126] Given the long history of associations between LINE elements and the synaptonemal complex, and their possible role in facilitating and directing synaptonemal complex assembly, this is an exciting area of research that needs to be more fully explored.[127]

Mutations in synaptonemal complex protein genes have severe implications for meiotic progression in murine systems. Mutations in SYCP2 and SYCP3 show a very similar phenotype, with a failure to form axial element structures and defective synapsis in spermatocytes, resulting in a block of prophase I around zygonema and male infertility.[84,115,117] Female *Sycp2* and *Sycp3* mutants, however, are both subfertile, having severely reduced litter sizes and a reduced reproductive life span.[115,117] SYCP3 protein is required for correct CO formation, and its absence increases nondisjunction in these mutant female oocytes.[117] *Sycp3* mutant females show increased aneuploidy, in which the abnormal karyotype is inherited by the offspring, often resulting in fetal demise, a situation that increases with advancing maternal age. *Sycp1* null males also exhibit a fertility defect as a consequence of large-scale asynapsis in prophase I.[128] In *Sycp1* null females, there is a complete absence of follicles in adolescent female ovaries; however, this can be partially alleviated by crossing to an *Sycp3* null background. This, along with other evidence, suggests that SYCP3 may play a role in meiotic checkpoint mechanisms in mice.[129,130] However, *Sycp1–Sycp3* double-null mice remain male and female infertile due to a lack of synapsis and a block of the DSB repair pathway (discussed further in this chapter) prior to CO resolution. Interestingly, these mice are still able to load cohesins onto the chromosome cores (also discussed further), even in the complete absence of a functional synaptonemal complex.[129]

In central element–defective mice, such as those with homozygous mutations in *Syce1*, *Syce2*, and *Syce3*, severe defects in synapsis and DSB repair (discussed further here) are evident, despite an apparently "normal" loading of SYCP1, resulting in complete infertility in both sexes. This suggests that although SYCP1 can load onto the axial element, assembly of the central element is impaired and thus synapsis cannot be achieved.[123,131,132] Interestingly, SYCE2 interacts with the recombination repair protein RAD51 (also discussed further here), indicating that sites of RAD51 localization might be candidates for synapsis initiation sites in the mouse.[132]

In addition to appropriate synaptonemal complex formation, there is an additional requirement to monitor the regulation of homolog interactions in the mouse. HORMA (Hop1p, Rev7p, and MAD2) domain-containing proteins, HORMAD1 and HORMAD2, localize selectively along unsynapsed chromosomes in wild-type mice, and dissociate upon accumulation of central element proteins. This reciprocal distribution of HORMAD and central element proteins is not dependent upon DSB repair components, but is absolutely dependent upon the presence of a functional synaptonemal complex, in addition to the AAA

(adenosinetriphosphatases (ATPases) associated with diverse cellular activities)–ATPase, thyroid hormone receptor interactor 13 (TRIP13).[133,134] HORMAD2 is also required for the accumulation of checkpoint kinase ATR (ataxia telangiectasia and Rad3-related protein) along unsynapsed cores in prophase I, as *Hormad2*$^{-/-}$ mice show defective ATR accumulation along the synaptonemal complex, suggesting that the HORMAD proteins play a major role in the meiotic prophase I asynapsis surveillance mechanism in mice (discussed further here).[135]

Very little is known about the synaptonemal complex disassembly process in mammals, but the beginning of the transition from prophase I to metaphase I involves both synaptonemal complex disassembly and chromatin compaction. During diplonema, the central element begins to disassemble, SYCP1 is lost from the chromosome cores, and the homologs repel each other[136] (Figure 1.7(D)). HORMADs relocalize to the cores upon desynapsis, while SYCP3, although remaining associated with the cores, begins to redistribute to the centromeres. Thus, the chromosomes are still held together by the synaptonemal complex at the centromeres and CO sites, and by the physical COs themselves, continuing into diakinesis[133,137,138] (Figure 1.7(E)). It is thought that central element disassembly begins with phosphorylation of SYCP1 and TEX12 by polo-like kinase 1 (PLK1), which facilitates their removal from the synaptonemal complex.[139] Subsequently, SYCP3 removal and lateral element reconfiguration processes are regulated by both the cyclin-dependent kinases (CDKs) and the aurora kinases (AURKs).[140,141] Further evidence to support the role of phosphatases and kinases in the transition process is that the phosphatase inhibitor okadaic acid can speed the G2–M transition in meiotic prophase cells.[142] SYCP1 remains localized to the centromeres until such time as meiotic kinetochores localize to the centromeres, consistent with a role for SYCP1 in assembly of these complexes in meiosis.[138] SYCP3 remains bound to the centromeres into diakinesis, where it promotes bi-orientation of homologous centromeres to help ensure proper segregation.[138]

Proper synaptonemal complex assembly and disassembly are critical steps in achieving appropriate DSB repair and subsequent correct meiotic segregation of chromosomes. This is true of humans as well as laboratory mouse models, and cases of mutation in synaptonemal complex component genes, such as *SYCE1* and *SYCP3*, have been implicated in women with premature ovarian failure and recurrent pregnancy loss.[143,144,145] Ectopic expression of synaptonemal complex genes has also been implicated in some rare incidences of cancer, demonstrating that meiosis-specific expression of these genes is crucial.[146]

Cohesins: Establishment and Assembly

As in mitosis, to ensure proper meiotic progression and correct chromosome segregation, sister chromatid cohesion needs to be established early in meiosis, and must be maintained until such time as the homologs and the sister chromatids need to separate at anaphase I and II, respectively. Thus, while cohesion is not meiosis specific per se, there are features of cohesion during meiosis that are very different from those of mitotic cells. The cohesion complex is similar in both cell cycles, consisting of two SMC proteins that enclose the DNA in a hinge, and two non-SMC proteins, the kleisin and stromalin subunits[147] (Figure 1.9(A)). In mitosis in higher organisms, cohesin loads onto chromosomes during replication and remains tightly bound until prophase, when the bulk of this cohesin dissociates from the chromosomes. By anaphase, all the cohesin has been removed, and the sister chromatids separate.[148,149] The timely removal of this cohesin is crucial for the proper segregation of sister chromatids. Cohesin binds along the entire length of the chromosome arms, but is intensely bound at the centromere, the region that is responsible for tightly binding sister chromatids.[150,151] During meiosis, however, the pattern of cohesin binding is very different; cohesion is established by cohesins binding to the chromosome arms, resulting in cohesion of paired homologous chromosomes, whereas once the first meiotic division has taken place, the sister chromatids remain joined by centromeric cohesion only.[152,153] Once the cohesin at the centromeres is disassembled in meiosis II, the sister chromatids may separate, similar to mitotic division.

Cohesion during cell division is maintained by specific cohesin complexes, the somatic cohesins SMC1 and SMC2, joined by kleisin RAD21 bound to stromalin SA1–SA2 (Figure 1.9(B)). In addition, most organisms contain meiosis-specific cohesins (Figure 1.9(C)), including SMC1β, REC8, RAD21L, and STAG3, which can combine with the canonical cohesins SMC1α, SMC3, and RAD21 to form varying complexes specific to meiosis. These proteins can be immunoprecipitated together as distinct complexes,[154] suggesting the presence of these varied subunit complexes in meiosis.

The timing of assembly and disassembly of meiotic cohesins through prophase I is complicated, and little is known about it in mammals; however, evidence from other eukaryotes suggests that cohesin assembly is a multistep process.[155] Cohesin assembly in mouse meiocytes begins with the localization of meiotic kleisin REC8 to chromosomes slightly before the premeiotic S-phase, followed by loading of the SMC proteins, SMC1β and SMC3, and synaptonemal complex axial element proteins, SYCP2 and SYCP3.[156] REC8 then remains localized to the chromosome arms longer than either SMC1β or SMC3, or the axial element proteins SYCP2 and SYCP3

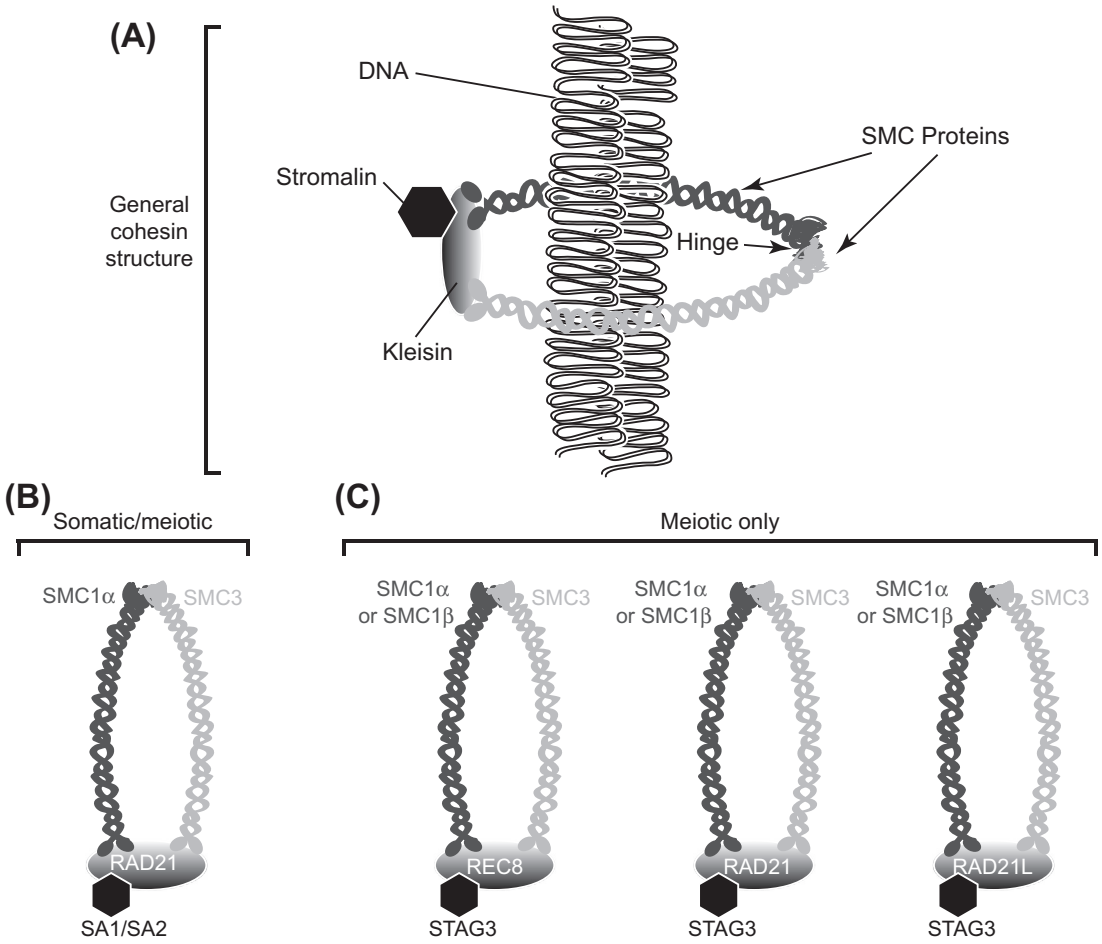

FIGURE 1.9 Cohesin structure in meiosis. (A) General cohesion structure consists of two SMC proteins (gray), which form a hinge around the DNA and are joined by a kleisin and stromalin subunit. (B) Somatic and meiotic cohesin. (C) Meiosis-specific cohesins.

(all of which dissociate from the arms by metaphase I, but remain localized at the centromeres) (Figure 1.7(F)). By anaphase I, REC8 is disassembled from the arms by separase cleavage, but is retained strongly at the centromeres. During meiosis II, all these proteins are disassembled from the centromeres simultaneously at anaphase II.[152,156,157] *Rec8* null mice are male and female infertile, with a complete arrest of spermatogenesis at meiotic prophase I, resulting from a failure to properly synapse. *Rec8* mutants do establish sister chromatid cohesion and accumulate synaptonemal complex proteins such as SYCP3 and SYCP1; however, the "synapsis" that they undergo is usually between sister chromatids, and thus they tend to suffer from precocious separation of sister chromatids, resulting in arrest at pachynema.[158,159]

A second kleisin subunit, RAD21L, also loads onto meiotic chromosomes early in leptonema, co-localizing with axial element proteins,[154,160,161,162] and it remains localized to the synaptonemal complex until pachynema or mid-diplonema, with strong staining on the axes of the X and Y chromosomes in males. Co-staining with REC8 in meiotic cells reveals mutually exclusive staining of these two kleisin complexes, suggesting that REC8 and RAD21L might form distinct cohesin-enriched domains along the axial elements, which consequently would form a unique pattern along the axial elements of chromosomes, resulting in a "barcode" that has been hypothesized to aid in homolog recognition.[154]

The third meiotic kleisin, RAD21, does not follow a localization pattern that is similar to those of REC8 and RAD21L. Instead, it tends to disappear during leptonema and zygonema, before reappearing in pachynema as RAD21L is dissociating from the cores, suggesting that it may be replacing RAD21L (and to some extent REC8, although the timing of REC8 removal from chromosome cores is not certain from data collected in several studies[154,156,157]). By metaphase I, RAD21 has also disassembled from the arms and remains only at the centromeres, similar to other cohesin localization patterns at this stage.[162,163,164] Given this differing localization pattern, it is thought that RAD21 may have a role in stabilizing sister chromatid cohesion at the time of DSB repair (during pachynema[165,166,167]), whereas REC8 and RAD21L may

have a more canonical role in sister chromatid cohesion during early prophase I. It appears that REC8 may be the basis for the loading of cohesin complexes at this time, given that REC8 loads onto chromosomes before any other cohesin or synaptonemal complex proteins,[156] and that REC8 remains bound to chromosome arms longer than the other cohesins, therefore being the only means by which cohesion is maintained until anaphase I. *Rad21l* mutant mice exhibit total male sterility, resulting from a lack of synapsis in prophase I and subsequent meiotic arrest at zygonema, reminiscent of the situation in *Rec8* mutants, whereas females exhibit age-related problems with fertility, most likely due to premature depletion of the oocyte pool, as is common with other female meiotic cohesin mutants.[162,168,169]

In mammals, the meiosis-specific stromalin subunit of cohesin, stromalin antigen 3 (STAG3), and the meiotic SMC proteins, SMC1β and SMC3, all localize in early leptonema of prophase I to the axial element in mammalian oocytes,[164,170] until late in prophase I, when they disassemble from the chromosome arms. The disassembly of these cohesins from chromosome arms appears to be dependent on kinases, such as NEK1.[171] STAG3 is removed from the chromosome arms at or around metaphase I,[172] as are SMC3 and SMC1β.[173,174] SMC1β remains localized to centromeres until metaphase II, presumably to retain sister chromatid cohesion until separation of the sister chromatids at meiosis II.[173] *Stag3* mutant rats exhibit sterility, presumably as a result of the pachytene arrest due to loss of STAG3 protein; however, this mutation has not been fully characterized, nor has *Stag3* been knocked out in a mouse model to date.[175] *Smc1β* mutant males exhibit pachytene arrest of spermatocytes due to incomplete synapsis, whereas in the mutant females, oocytes progress to metaphase II. Axial elements in the mutants are shorter, and the chromatin extends further from the core, suggesting that SMC1β is involved in chromatin loop formation and organization.[85,176,177] In addition, in these mutants, CO structures and meiosis-specific telomere adjustments are absent. This, along with other evidence, indicates that SMC1β plays an important role, both in maintenance of telomeres during meiosis and as a chiasma-binding factor that stabilizes CO structures until anaphase I.[85,178] This is important evidence toward providing a link between axial element length and chromatin loop size with cohesin components. STAG2 (similar to mitotic stromalin (stromalin 2, or SA2)) has also been implicated in meiosis, although it is unclear in which complex it functions; it might be functionally associated with RAD21, as the localization patterns remain the same, and may cooperate with STAG3 complexes, although very little is known about its function in meiosis.[179]

Phylogenetic analyses have identified two further proteins belonging to the SMC family,[180] known as SMC5 and SMC6. In mammals, these proteins are highly expressed in the testes, and are associated with the XY chromosomes during prophase I of meiosis.[181] Very little is known about the role of these SMCs in mammalian meiosis; however, they have been implicated in repair processes in human somatic cells[182] and joint molecule resolution in yeast, not all of which are formed as a result of SPO11-dependent DNA breaks (discussed further in this chapter).[183,184] They do not appear to function in the cohesion-like processes for which other family members are best known. Other cohesin proteins have been implicated in meiotic repair pathways in many eukaryotic organisms, such as mouse, *C. elegans*, *D. melanogaster*, and *Arabidopsis thaliana* (mouse,[178] *C. elegans*,[185] *Drosophila*,[186] and *Arabidopsis*[187]), and in somatic repair.[165,188,189]

Meiotic Recombination

Overview of Meiotic Recombination

As described in this chapter, one of the ways in which homologous chromosomes can remain physically paired until the first meiotic division is via DNA:DNA interactions between maternal and paternal genomes. To do this, homologous chromosomes must break and interhomolog DNA connections must form. This process is known as crossing over, or reciprocal recombination, since there is a reciprocal exchange of DNA between maternal and paternal chromosomes (because exactly the same length of DNA must be swapped). Therefore, crossing over is the second fundamental process of prophase I and involves the exchange (or recombination) of genetic information between homologous (maternal and paternal) chromosomes.

In almost all species examined to date, crossing over is initiated by the formation of a DSB in one sister chromatid of one homologous chromosome during leptonema. Thus, while most cells of an organism must actively avoid DNA breaks, meiotic cells undergo genetically programmed breaks that must then be repaired in a specific way that is extremely tightly controlled both temporally and spatially. The formation of a DSB in leptonema and its subsequent repair through prophase I are called homologous recombination, which serves three crucial functions during prophase I. First, the exchange of genetic material between homologs generates diversity within gametes by shuffling the DNA and introducing variation; second, the physical events of recombination allow for homology searching between chromosomes (explained further here); and, third, and arguably most importantly, it creates the physical manifestations of crossing over, the chiasmata, that hold homologous chromosomes together at the metaphase plate during meiosis I, thus reducing the risk of

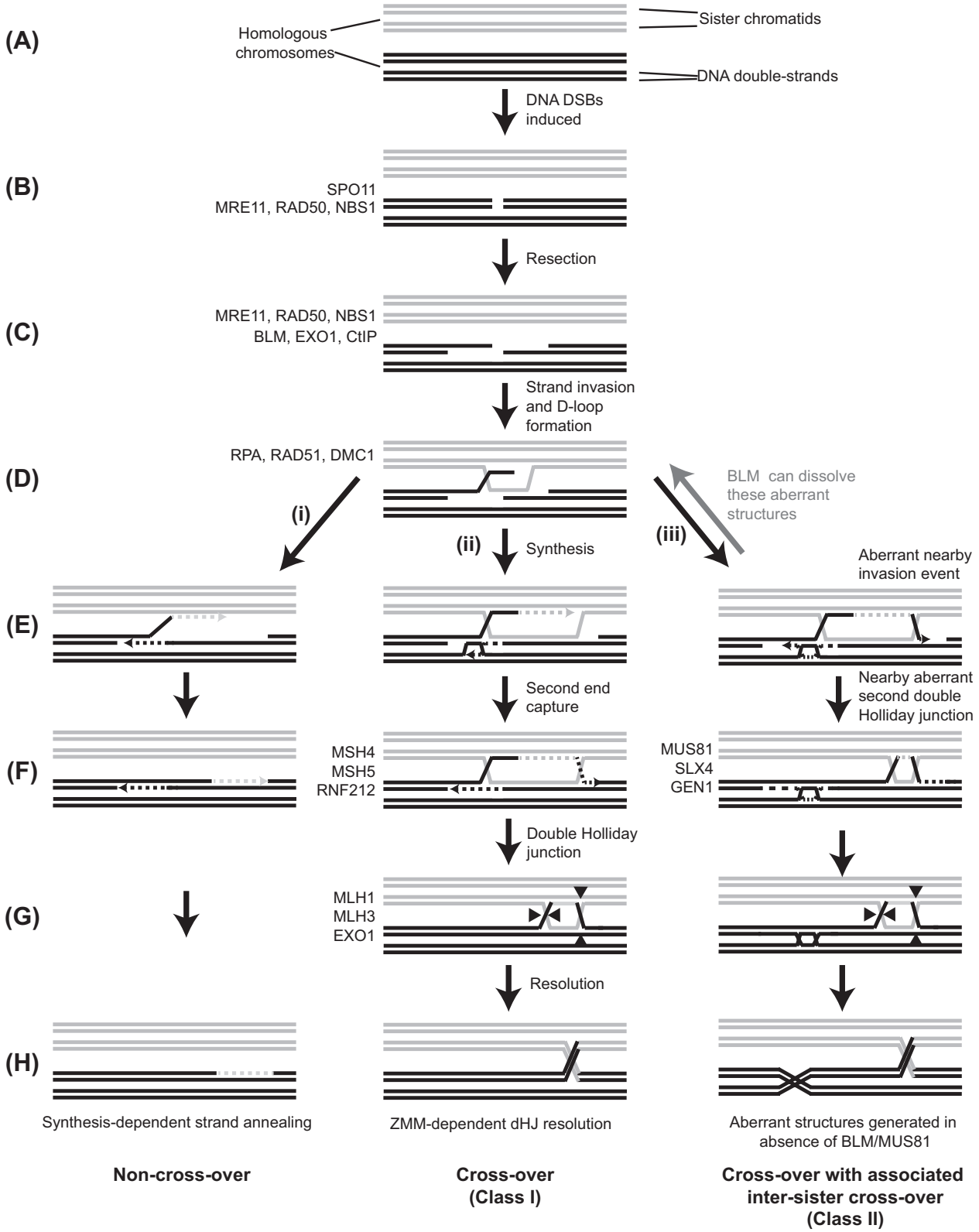

FIGURE 1.10 **Double-strand break repair model in eukaryotes.** A schematic representation of double-strand break repair (DSBR) in eukaryotic meiosis. (A) Homologous chromosomes (gray and black) undergo DSBR during meiotic prophase I. (B) SPO11 and its accessory factors form a DSB in the DNA of one homolog (in this case, the black one), which can then be repaired by one of several pathways: either as a non-crossover (left panel), as a class I crossover (middle panel), or as a class II crossover (right panel). (C) The cut DNA ends are resected back by exonucleases to form free 3′ overhanging strands. (D) These 3′ overhangs (black) may "invade" the opposing homologous partner (gray) to form a D-loop structure. (E) Depending on the method of repair, the D-loop can form several intermediate structures, and the DSB can be repaired in a number of ways. (F–H) Non-crossovers are thought to be repaired by a process known as synthesis-dependent strand annealing (SDSA, left panel, pathway i), during which no classic double

aneuploidy in future gametes. This third requirement also demands that CO are placed at defined points throughout the genome, with at least one CO exchange event occurring on each chromosome, and with the placement of one CO limiting the placement of nearby CO events. Thus, as described here, the repair of DSBs is extremely tightly regulated at all stages of recombination.

DSB repair is extremely complex, and it is described in detail in this chapter. Briefly, however, the chromatid that is broken must be processed to allow one end of the break to become invasive. This invading end can then insert into other DNA strands to "test" them for homology (by simple base pairing) and to initiate homolog interactions. Once the appropriate homolog is located, these DSBs must then be repaired, either by forming hybrid DNA molecules with the opposing homologous sequence or by reannealing to the other end of DSB. Multiple DNA repair mechanisms exist for these purposes, many of which are similar between meiotic and mitotic cells, but which have been selectively "repurposed" to allow for the formation of distinct CO structures between homologous chromosomes. It is important to note, therefore, that DSB repair is not confined to meiosis, but also occurs in response to spontaneous (unprogrammed) DSBs that can arise in somatic cells. The molecular events of DSB repair in gametes are reminiscent of those in somatic cells, with a few major differences, as detailed in this chapter. Due to the problematic nature of studying these processes in detail in mammals, most of what is known about the mechanisms of meiotic DSB repair comes from research in yeast; however, due to the highly conserved nature of most meiotic processes, much of this data has been extrapolated to mammals. In fact, many of the fundamental steps of DSB repair involve proteins that are highly conserved across species, from yeast to humans.

Physical Events in Meiotic Recombination

The term "recombination" encompasses a number of DNA modification events that occur during prophase I, all of which are initiated by the induction of a DSB into the DNA, creating a blunt end nick in one sister chromatid of a recently replicated sister chromatid pair. The DSB is processed initially by resection of the two 5′ ends (Figure 1.10(C)), which results in 3′ single-stranded overhangs that are then capable of invading an opposing homolog and, in doing so, displace a stretch of DNA from the opposing homolog, known as the D-loop (Figure 1.10(D)). The D-loop may be repaired by several mechanisms, depending on the nature of the initial strand invasion event, the placement along the chromosome, and other factors. In the case of most CO events, the D-loop becomes further processed to form a structure known as a double Holliday junction (dHJ): (1) DNA synthesis from the end of the invading overhang (having lost a portion of DNA sequence due to the original resection event), using the opposing homolog as a template, stabilizes a "single end invasion" intermediate[190]; (2) subsequent religation of the invading strand with its original 3′ end results in what is termed "second end capture"; and (3) this occurs in tandem with DNA synthesis from the noninvading 3′ overhang, using the displaced D-loop as a template, followed by religation, all resulting in the formation of two stretches of heteroduplex DNA within what now is known as a dHJ (Figure 1.10(E), pathway (ii)).[191] Repair of this structure can give rise to COs, in which a flanking DNA is reciprocally exchanged, or non-crossovers (NCOs). However, for reasons that are not entirely apparent, dHJ repair in meiosis is heavily biased toward the formation of COs (discussed further here).

COs may also form from structures other than dHJs in yeast, but these have yet to be observed in mammals. In addition, it is possible for nearby DSB repair events and/or events that involve multiple-strand invasions from a single DSB to result in recombination intermediate structures involving all four chromatids (Figure 1.10(E)–(H), pathways (iii)). These structures, termed "multichromatid joint molecules" in *S. cerevisiae*, can be repaired in a number of ways, but appear to be rare events that are processed through specialized machinery.

As will be discussed here, the majority of DSBs do not become COs and instead are processed through NCO pathways, giving rise to repair events that do not involve exchange of parental genetic information. Given the bias of dHJ repair in meiosis toward COs, it appears that NCOs are achieved by temporally distinct mechanisms that arise during strand invasion and/or D-loop formation. For example, in *S. cerevisiae*, NCOs are obtained from DSB repair through a process called the synthesis-dependent strand annealing (SDSA) pathway, in which the invading strand is ejected from the D-loop and religates to its own 5′ end with the assistance of DNA synthesis using either the sister chromatid or the opposing homolog as the template, the latter resulting in a gene conversion event (Figure 1.10(E)–(H), pathways (i)).

◀ Holliday junction (dHJ) is formed (F, left panel). Conversely, class I crossovers (middle panel, pathway ii) are thought to be formed by the formation and repair of a dHJ (F, middle panel). Class II crossovers are seemingly more complex (right panel, pathway iii), perhaps being constructed as a result of repairing aberrant joint molecule structures (F,G, right panel) that arise too close to class I crossovers, or as a result of multiple invasion events during DSB repair, resulting in closely spaced crossover products (right panel, H). (F–H) Non-crossovers are thought to be repaired by a process known as synthesis-dependent strand annealing (SDSA; see left panel, pathway (i)), during which no classic double Holliday junction (dHJ) is formed (F, left panel). Conversely, class I crossovers (middle panel, pathway (ii)) are thought to be formed by the formation and repair of a dHJ (F, middle panel). Class II crossovers are seemingly more complex (right panel, pathway (iii)), perhaps being constructed as a result of repairing aberrant joint molecule structures (F, G, right panel) that arise too close to class I crossovers, or as a result of multiple invasion events during DSBR repair, resulting in closely spaced crossover products (right panel, H).

An intriguing aspect of meiotic recombination is the fact that many more DSBs are formed than are actually processed into COs. This has prompted the conclusion that recombination serves multiple purposes beyond those that involve reciprocal recombination. In other words, physical tethering of homologous chromosomes through crossing over represents just one function of recombination. Indeed, in mammals, approximately 10-fold more DSBs are created than are processed into COs, with the remaining DSBs being repaired either through intersister chromatid interactions or through NCOs,[192] neither of which contribute to homolog segregation because they do not involve the formation of heteroduplex DNA between the homologous chromosomes. They may, however, contribute to the processes of homology searching and recognition since their repair progression includes initial strand invasion.

Initiation of DSBs

DSBs are introduced in high numbers in leptonema by the topoisomerase SPO11, an enzyme that introduces an asymmetric break in the DNA strand (Figure 1.10(B)). *Spo11* is expressed predominantly in the testes and ovaries of mice, although real-time polymerase chain reaction studies have found expression in certain somatic tissues in humans.[193,194,195,196] SPO11 protein is found in early prophase I, during leptonema, and mice lacking a functional *Spo11* gene show severe defects in prophase I progression. These defects include a complete absence of synapsis and a lack of DSBs, resulting in complete infertility of both male and female mice.[193,194,197,198,199] DSB induction and at least partial prophase I progression can be restored by cisplatin treatment, which induces DNA lesions that are similar to those created by SPO11.[199] Loss of *Spo11* in these mice also affects the downstream expression of several other genes involved in DSB initiation and processing, such as *Hop2*, *Brca2*, *Mnd1*, and *FancG*.[200] Importantly, the synapsis defects in *Spo11* mutant mice, and indeed in many mutants that disrupt DSB repair, demonstrate the requirement for recombination events to ensure homolog recognition and the ensuing pairing events.

In wild-type mice, SPO11 protein is expressed as two separate isoforms as a result of alternate splicing of exon 2, producing an exon 2-skipped isoform, SPO11α, and an exon 2-containing isoform, SPO11β. *Spo11β* is expressed from the onset of prophase I in juvenile male mice, whereas *Spo11α* is expressed from pachynema and is responsible for the unique, temporally distinct COs that form on the XY chromosomes (discussed further in this chapter). Both isoforms are present in female meiosis, although female mice expressing the *Spo11β*-only isoform are fertile, while their male counterparts are sterile, indicating this α isoform does not play a major role in female prophase I progression, which is expected given that its role in male meiosis is restricted to the X and Y chromosomes.[195,196,201,202]

Spo11 does not act alone; in yeast, it is known to require at least nine accessory proteins, both to localize Spo11 appropriately and to facilitate DSB formation (Figure 1.10(B); reviewed in Ref. 203). The catalytic activity of Spo11 comprises a nucleolytic attack of the DNA, resulting in a 5′ phosphotyrosyl bond and a 3′ hydroxyl, and Spo11 protein remains bound to the DNA ends until it is actively removed. It is these Spo11-bound DNA ends, or oligos, that are the basis for recent deep sequencing approaches that have identified Spo11-binding sites in the yeast genome.[204] This cleavage activity requires the MRX complex (MRX: Mre11, Rad50, and Xrs2), which is also known as the MRN complex in mammals (MRE11, RAD50, and NBS1).[205,206,207] The MRX complex works in conjunction with its partner Sae2–Ctp1 (reviewed in Refs 208,209), as well as several other factors such as Mei4, Rec102, Rec104, Rec114, Mer2, and Ski8, a subset of which have been identified in mammals as MEI4 and REC114[210] (and are reviewed in Ref. 203). MEI4 and REC114 have been shown to functionally interact in the mouse, and localize to meiotic chromosomes in early prophase I.[210] *Mei4*$^{-/-}$ mice show defective DSB formation, leading to infertility, indicating a role for these proteins similar to that of their yeast homologs, and of mammalian SPO11, in facilitating DSB formation.[210] A separate gene, *Mei1*, which is not conserved in yeast, has been identified by forward genetic screen to be crucial for DSB formation in mice.[211]

Previously, it was assumed that SPO11-induced DSBs were generated randomly throughout the genome and, while the presence of CO homeostatic mechanisms such as interference (discussed further in this chapter) dispelled this myth, only recently has it been possible to demonstrate the lack of randomness at the nucleotide level through next-generation sequencing methodologies. Recent advances in the mapping of DSB sites, along with techniques such as SPO11-associated oligo mapping (described here) in yeast and mouse, have allowed for a new understanding of how DSBs are targeted to specific genomic locations. Indeed, DSBs appear to be preferentially induced at very specific locations, termed DSB initiation hotspots, where recombination occurs at rates that are many thousandfold higher than the genome average.[196,212,213,214] Both the genomic map gleaned from the positions of single-stranded ends processed after DSB processing, and the more specific map extracted from sequencing Spo11 oligos, align very well (reviewed in Ref. 215). In agreement with many previous studies showing the preference for hotspot locations, the vast majority of Spo11 oligos map to nonrepetitive elements, mainly gene promoters, and GC-rich regions of the genome, both features associated

with a more accessible chromatin structure, and where Spo11 can presumably more easily act on the DNA. SPO11-associated oligos have been extracted from mice also, although mapping of these has not yet been performed in detail.[196]

Initial DSB Processing

Following DSB formation, DNA resection takes place to leave single-stranded DNA overhangs. In budding yeast, resection initiation depends upon Sae2 protein and is severely hindered in the absence of Mre11 and Exo1.[216,217,218] Recent studies in yeast have determined that this resection is also bidirectional, with Mre11 first nicking the DNA several hundred bases away from the 5′ terminus of the DSB, and then resection occurring by Mre11 in a 3′ to 5′ direction toward the DSB, while Exo1 mediates 5′ to 3′ resection away from the DSB (Figure 1.10(C)).[217] In mammals, orthologs of these genes, *Mre11*, *CtIP* (the *Sae2* ortholog), and *Exo1*, are all required for meiotic prophase I progression. *Mre11* hypomorphic mice showed high levels of asynapsis of homologous chromosomes, with a different effect on male (increased) and female (decreased) frequency of MLH1 foci (indicative of nascent COs), whereas *Exo1* null mice progress apparently normally until diakinesis, where they lack sufficient chiasmata to progress further. This later phenotype of *Exo1* null mice might be due to a distinct role for EXO1 in promoting CO formation, entirely independent of its putative early prophase I role as an exonuclease, similar to that seen in yeast and other organisms[219,220,221,222] (discussed further here). Other factors, such as yeast Dna2, are required for mitotic DNA resection at sites of repair, but as yet, very little is known about their function in mammalian meiosis.[223]

Following resection, the single-stranded DNA (ssDNA) becomes coated in replication protein A (RPA), which prevents the ssDNA from looping back on itself and forming aberrant secondary structures (reviewed in Ref. 224). In mice, RPA localizes to meiotic chromosome cores, coincident with DSB sites.[225,226] ATM colocalizes with RPA along meiotic chromosomes, indicating a possible functional interaction between the two proteins.[225] The ssDNA then loads the RecA homologs, RAD51 and the meiosis-specific DMC1, both of which physically interact with RPA in human cells and may be loaded onto chromosome cores by the TEX15 protein.[226,227,228,229,230] Together, RAD51 and DMC1 facilitate invasion of an opposing homolog by the ssDNA end, which displaces one strand of the recipient homolog to form the D-loop, while in mitotic cells, RAD51 performs a similar role without the aid of DMC1. Both RAD51 and DMC1 localize at meiotic DSB sites in mammals, at a frequency of around 250 per nucleus in the mouse, beginning by associating with single cores but later lying between homologous cores as synapsis proceeds, a process that may be facilitated by the RAD51 associated protein 1 (RAD51AP1; Figure 1.7(H)). RAD51AP1 enhances the ability of DMC1 to capture the duplex DNA homologous partner and may mediate interactions between DMC1 and RAD51 in meiosis.[227,231,232] *Dmc1* mouse mutant spermatocytes fail to proceed past pachynema and both males and females are completely sterile (Figure 1.11(G)), indicating that DMC1 protein is absolutely required for proper meiotic DSB repair.[233]

Recent data suggest that D-loops coated with human RAD51 in vitro are more easily dissociated by factors such as RAD54 than those coated with DMC1 protein, and thus are more susceptible to repair as NCOs by SDSA (Figure 1.10, pathway (i)), suggesting that DMC1 might localize to D-loops destined to be repaired as COs, while sites designated as NCOs accumulate RAD51.[234] This is supported by recent evidence showing that the joint molecule (JM) function of Rad51 is completely dispensable for CO formation in yeast, while CO formation is entirely dependent on the JM function of Dmc1.[235] It is also known from yeast studies that Dmc1 and Rad51 are loaded using separate protein complexes and pathways, although these may differ from organism to organism, but are loaded in a side-by-side pattern on meiotic chromosomes.[236,237,238] Further evidence from *A. thaliana* suggests that DMC1 and RAD51 have functionally and spatially distinct roles, and each localizes to the opposite side of a DSB.[239] Additionally, Rad51 filaments are thought to be dissociated from D-loops in yeast by Srs2 protein, and indeed, *SRS2* mutants display an increase in COs, suggesting that due to RAD51 nonremoval from the D-loops, more COs are formed in the absence of Srs2.[240,241] In the mouse, RAD51 and DMC1 localize to the nucleus during leptonema, usually in large numbers of 250–300 foci per cell (Figure 1.7(H)),[242] but little is known regarding the preferential binding of these proteins for NCO and CO events, and it has been assumed until recently that the two proteins function in concert.

The serine–threonine kinases, ATM and ATR, master regulators of the ubiquitous DNA damage response, have been shown to play a major role in meiotic prophase in mammals: by localization studies showing they are present on meiotic cores, by mutational analysis showing that ATM is directly required for meiotic progression in mice, and by recent data showing that ATM is responsible for regulating the number of SPO11-induced DSBs in meiosis via a negative-feedback loop, in which DSB formation induces ATM expression and/or activation and ATM, in turn, downregulates SPO11 activity.[243,244,245] Thus, spermatocytes lacking ATM show a 10-fold increase in SPO11-linked oligonucleotides, indicating that ATM regulates the frequency of DSBs. Conversely, mice bearing one mutant allele of *Spo11* (*Spo11*$^{+/-}$) and two mutant alleles of

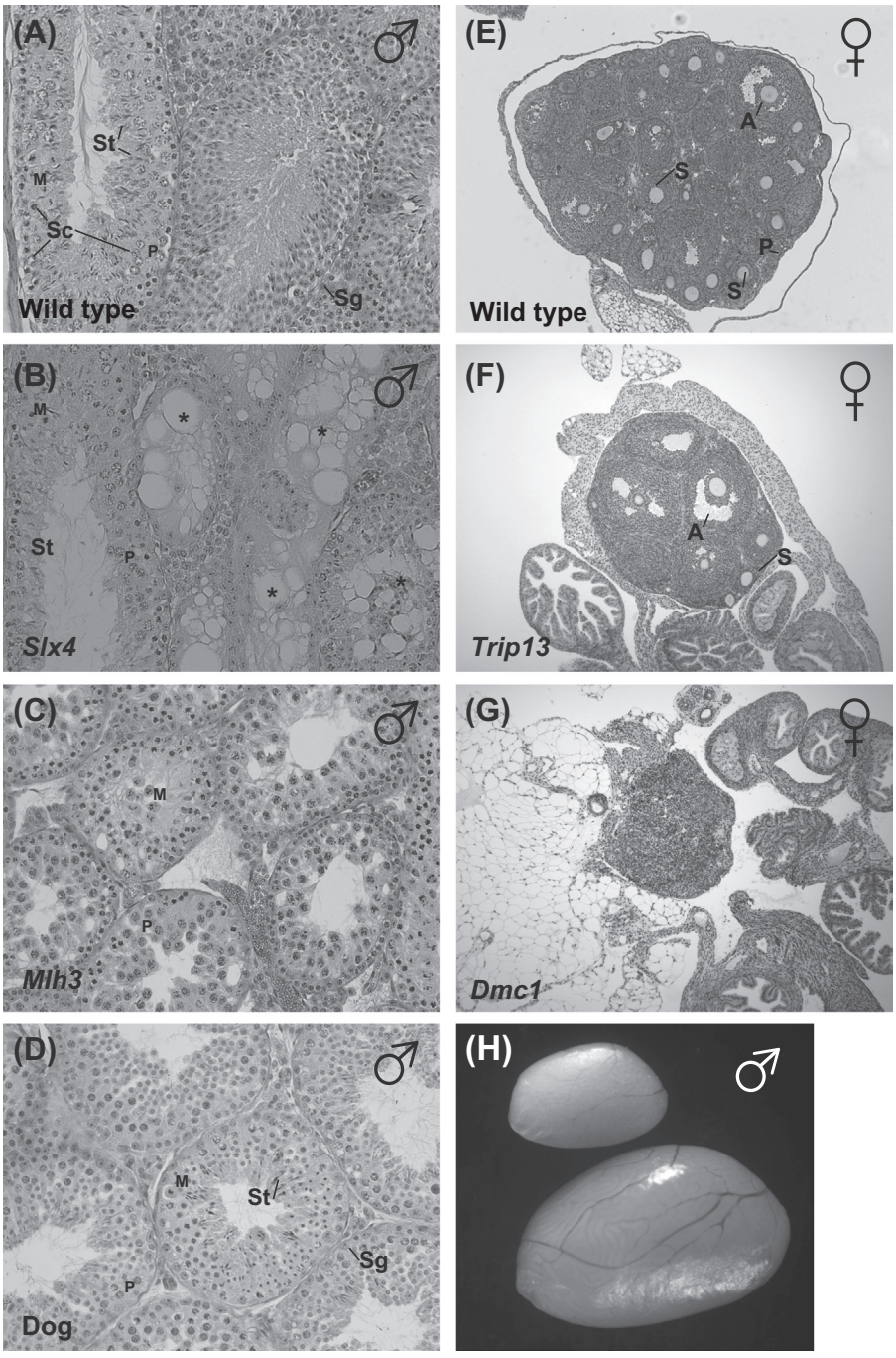

FIGURE 1.11 Phenotypic variation in meiotic mutants. (A–C) Testis sections from (A) wild type, (B) *Slx4* mutants, and (C) *Mlh3* mutants. Wild-type sections show a range of cell types from spermatogonia (Sg), to spermatocytes (Sc) in pachytene (P) and metaphase I (M) stages, to spermatids (St). *Slx4* mutants show these cell types in some tubules, but have other tubules completely devoid of meiotic cells (asterisk), indicative of a premeiotic stage error. *Mlh3* mutants show germ cells in every tubule, but no cells at a later stage than spermatocyte, indicating an arrest at the end of prophase I, due to meiotic errors. (D) Dog testis section shows a very similar morphology to that of the wild-type mice. (E–G) Ovaries from female mice that are (E) wild type, (F) *Trip13* mutant, and (G) *Dmc1* mutant. Wild-type ovaries show a mix of follicle stages, including primary (P), secondary (S), and antral (A). *Trip13* mutants show different stages of follicles, but vastly fewer in number in the whole ovary than wild type, and no primary follicles, indicating a premature loss of the oocyte pool. *Dmc1* mutants show no follicles at all, indicating they never made it past the very initial stages of meiosis before dying. (H) Size difference between testis of a wild-type male mouse and a meiotic mutant littermate (*Nek1* mutant).

Atm (*Atm1*$^{-/-}$) exhibit a less severe meiotic defect than do *Atm*$^{-/-}$ mice, suggesting a complex interdependent feedback loop between the two proteins. It is possible that ATM serves either to directly phosphorylate SPO11, or one of its accessory proteins, at DSB sites to prevent subsequent DSBs from forming in the same vicinity, or to restrict excessive DSB formation across the genome. ATM is functionally active at the sites of DSBs, given that one of its major targets, the histone H2AX, is phosphorylated at DSB sites in many organisms.[197,245,246] ATM–ATR homologs are also responsible for other important phosphorylation events during DNA repair, including phosphorylation of Sae2 in yeast meiotic DSB resection and phosphorylation of Rpa in mitotic yeast cells, and although these interactions might be true also in mammalian systems, little is known about their roles in meiosis.[247,248]

The Bloom's syndrome mutated (BLM) helicase also plays an important role in DSB repair in both meiotic and mitotic cells, and it has a critical role in meiotic progression in mice (as does its yeast homolog, Sgs1). BLM/Sgs1 is thought to act at sites of DSBs, where it localizes to chromosome cores in early prophase I, and also in the latter

stages, during which it is thought to play a similar role to that in yeast, that of: untangling aberrant recombination structures, along with MUS81–EME1 (discussed further in this chapter).[249,250,251,252,253,254] Mice with a conditional knockout of BLM helicase in their spermatocytes display errors in meiotic synapsis, as well as in the proper repair of DSB intermediates, resulting in higher levels of unrepaired DSB intermediates at diakinesis and an increase in chiasmata-like structures at metaphase I.[254] BLM has been implicated as a major component of the Class II CO pathway in mice (also discussed further here).

CO versus NCO Fates: The Role of the ZMM Proteins

The decision to become a CO or an NCO following the initial common intermediate steps of DSB repair is regulated by several processes, not least of which is the fact that each and every chromosome must receive at least one CO, and that these COs must be appropriately spaced to prevent aberrant recombination events and/or to reduce the risk of genome instability. Following D-loop formation, DSB repair intermediates may be processed through one of several pathways, resulting in the formation of NCOs or COs (Figure 1.10). Studies in *S. cerevisiae* have revealed that NCO events are resolved much earlier in prophase I than COs, pointing to different resolution pathways for the two recombinant products. Instead, a temporally distinct NCO pathway has been proposed for yeast: the SDSA pathway (Figure 1.10, pathway (i)). In this scenario, failure to capture the second end of the DSB (discussed further here) leads to displacement of the invading strand in the D-loop, and instead the two strands can anneal, resulting in an NCO with minimal gene conversion (Figure 1.10, pathway (i)).[255,256]

Originally, it was thought that processing of DSBs through the dHJ intermediate and the subsequent cutting and resolution of that dHJ via one of two possible orientations could result equally in either COs or NCOs.[257] However, through mechanisms that are not clearly understood, dHJ resolution is extremely biased toward the formation of COs.[258] Members of the so-called ZMM family of proteins are thought to mediate this bias, including in yeast the synaptonemal complex protein Zip3 (along with Zip1, Zip2, and Zip4), the DNA mismatch repair proteins MutS homolog 4 (MSH4) and MSH5, and the adenosine triphosphate (ATP)-dependent helicase Mer3. Mammals and other organisms possess orthologs of the ZMM proteins, including worm ZHP3 and mouse and human RNF212 (orthologs of Zip3); MSH4/5 in mouse, human, and worm; and mouse HFM1 (ortholog of Mer3).[259] The cyclin B interacting protein, HEI10 (or CCNB1IP1), present in mouse and *Arabidopsis*, has recently been added to the list of ZMM proteins.[260,261] These proteins function collectively to ensure that dHJ processing results only in COs, as demonstrated by the fact that COs are significantly reduced or entirely absent in mutants for each of the ZMM genes across eukaryotes (reviewed by Ref. 192).

The DNA Mismatch Repair Pathway Regulates the Major Pathway for Crossing Over

Second end capture results in the formation of a dHJ, which is stabilized by the meiosis-specific MutS homolog heterodimer of MSH4 and MSH5, known as MutSγ. Hydrolysis of ATP through the MutSγ ATPase domains allows the MSH4–MSH5 heterodimers to form a sliding clamp that may displace the complex from the immediate vicinity of the dHJ, facilitating further clamp loading.[262,263,264] In mice, MSH4 and MSH5 proteins localize in distinct foci along chromosome axes during zygonema of prophase I, usually in much higher numbers than the eventual number of COs in the nucleus, at around 150 per nucleus (Figure 1.7(I)). MSH4–MSH5 numbers gradually decline into pachynema, until by the mid-pachytene stage they number approximately 30 per nucleus.[226,265,266] Recent data have suggested that certain MSH4–MSH5 foci are designated from the entire pool of foci as future CO sites, and these sites are stabilized in the mouse spermatocyte by the Zip3 homolog RNF212.[267] In addition, data from *C. elegans* suggest that preselected CO sites are processed by the cyclin-related protein COSA1, which is recruited only to sites of CO. Both MSH4 and MSH5 are critical for proper meiotic progression in the mouse, and the loss of either protein results in the absence of COs and complete sterility in males and females.[265,266] In addition, they load with similar temporal and spatial frequency on synaptonemal complexes from human oocytes, indicating similar functions.[268]

The MutSγ heterodimer recruits a second MMR heterodimer, this time composed of the MutL homologs, MLH1 and MLH3 (MutLγ).[269,270,271,272] Surprisingly, the complex is recruited sequentially in pachynema, MLH3 appearing on prophase I chromosome cores slightly prior to MLH1 in mouse, and localizing to cores even in *Mlh1*$^{-/-}$ mutants, indicating that MLH3 can bind to DNA without MLH1 present.[273] MLH1 then proceeds to co-localize with MLH3 at all but a small subset of foci in mouse spermatocytes, thus marking the position of the Class I COs (Figure 1.7(J)).[273] This Class I pathway is well characterized in lower eukaryotes, such as *S. cerevisiae* and *A. thaliana*, but very little is known about the control mechanisms in mammals.[274,275] Studies of a well-characterized recombination hotspot in the mouse, *Psmb9*, as well as cytogenetic studies in mutants for *Mlh1* and *Mlh3* have indicated that 90–95% of COs depend upon the Class I, MLH1–MLH3-dependent, pathway, while the remaining COs are processed through a Class II pathway that is regulated by the MUS81–EME1 endonuclease (discussed further in this chapter).[273,276,277,278] Additional proteins localize to sites of Class I COs in the

mouse, and are crucial for proper progression of meiosis in the mouse. These include the cyclin-dependent kinase CDK2, which co-localizes with MLH1 in spermatocytes, and in which a mutation can cause infertility in the mouse; RAD51C, which localizes to sites of CO in late prophase I, is critical for meiotic progression in mice, and is required for HJ processing in somatic cells; and EXO1, which has been shown to have a crucial late role in CO processing following MLH1–MLH3 accumulation in the mouse, with *Exo1*$^{-/-}$ mice accumulating MLH1–MLH3 normally, but then having very few COs, leading to sterility.[221,279,280,281,282] Class I-processed dHJs are resolved into COs late in prophase I, by as yet unidentified resolvases. Many proteins have been implicated in this role in both mammals and lower eukaryotes, including MUS81, MLH3, EXO1, YEN1, and SLX4; however, no definitive single resolvase has yet been discovered.[283,284]

A Second Class of COs that are Independent of MutLγ

A second class of CO is thought to generate about 10% of COs in mammalian gametogenesis, given that *Mlh1* or *Mlh3* mutant mice have two to three residual chiasmata per cell and retain about 10% of total COs at hotspots.[270,272,276,277] These COs are not regulated by the ZMM proteins, and are instead thought to be dependent on the structure specific endonuclease, MUS81, because in *Mus81* mutant mouse spermatocytes the number of MLH1 foci increases in pachynema; however, the total number of chiasmata at diakinesis remains the same, indicating that the MLH1–MLH3 Class I CO pathway somehow compensates for the loss of MUS81.[278] In yeast, it is thought that Mus81 acts in concert with Sgs1 (the BLM helicase yeast homolog) to disassemble aberrant joint molecule structures that occur as a result of improper stand invasion events during DSB repair.[250,251,252,253] This may be true in mammals also, as mice displaying a testis-specific conditional knockout of *Blm* show an increase in chiasmata-like structures at diakinesis.[254] It might be the case that perhaps many resolvases are present to generate both classes of CO in meiosis, and that they have overlapping and compensatory roles in processing intermediates, which certainly appears to be the case in yeast, where several of these components can be removed and COs still processed.[222,285]

Multistage Regulation of CO Placement and Frequency through Prophase I

It is clear that the frequency and location of DSB induction in mice are tightly controlled. One example of their regulation was presented here, namely, the feedback control on Spo11 activity in both yeast and mouse by the ATM kinase.[245] Recent evidence has shown that DSB position is also tightly controlled, being preferentially initiated at regions that correspond to testis-specific trimethylation of lysine 4 on histone H3.[286] This methylation mark is a result of binding and methyltransferase activity by the zinc finger protein, PRDM9.[287,288,289] PRDM9 is thought to trimethylate histones upon binding to specific recognition sequences of the genome, which in turn attracts SPO11 to these hotspot sites.[286,290] DSBs occurring in *Prdm9* mutant mouse spermatocytes are not preferentially biased toward hotspots, but instead arise at alternative regions of the genome that have H3K4 trimethylation marks, such as gene promoters.[291] The formation of these DSBs is also kinetically slower than that in wild-type mice. PRDM9 is highly specific in its recognition of DNA sequence, and slight changes in the highly variable zinc finger motif render it incapable of binding certain alleles and more likely to bind elsewhere. Indeed, in humans, individuals with differing PRDM9 alleles can use entirely different sets of CO hotspots, raising a key issue known as the hotspot paradox.[292,293] This paradox is borne out of the fact that any PRDM9 allele that is active receives a DSB preferentially, while a nonactive allele does not. Due to the nature of dHJ formation, which by necessity includes repair of heteroduplex DNA, the intact homolog, which is inactive for hotspot activity, is used as a template for repair, effectively inactivating the recombination hotspot at this location through the process of gene conversion. This process, it is hypothesized, could eventually lead to the extinction of this initiating allele from a population, unless mechanisms (yet to be determined) exist to preserve the hotspot activity.[294,295]

Studies in a wide range of mammals have confirmed that PRDM9 is a global regulator of meiotic recombination in mammalian species. Intriguingly, though, PRDM9 is completely absent from dogs and their related species, with all canids showing a significantly different recombination landscape across the genome from that seen in humans and mice. These data indicate that there is an alternative pathway available for generating the recombination landscape across the mammalian genome.[291,296,297]

In yeast and mice, an interesting phenomenon occurs, by which CO frequency is maintained, even in the presence of fewer initiating DSB events, and is termed CO homeostasis.[298] This process ensures that, even in the case of fewer Spo11-induced DSBs, the final tally of COs is maintained, ensuring one CO per chromosome, or obligate CO. CO homeostasis is demonstrated in mice by the fact that number of DSBs induced during early prophase I far exceeds the number of eventual COs that result from repair of these breaks and that, although DSB numbers can change, final CO is exceptionally tightly controlled.[299] For example, an early marker for DSB repair is RAD51. The number of RAD51 foci in early zygonema is 100–500 per nucleus, with an average of around 219 foci per zygotene nucleus. A somewhat later (zygotene–pachytene) marker, MSH4, shows lower

focus numbers per nucleus in the mouse, around 100–200, with the difference being accounted for by NCO mechanisms. Finally, the late pachytene marker MLH1 shows many fewer foci, in the region of 20–25,[270,300] again the balance being made up of DSB repair events that are destined toward NCO pathways. Not only does the number of foci for each of these recombination markers decline as prophase I progresses, but the regulation of marker frequency becomes increasingly stringent also, with lower variability in focus frequency, and steadily decreasing coefficients of variation in focus numbers as we proceed from zygonema to pachynema. The final tally of MLH1 foci in late pachynema is extremely conserved, with very, very low variability between nuclei. Even if DSB frequency is varied by changing the dosage of SPO11 protein in mice, the eventual number of MLH1 foci remains surprisingly unchanged, with the variable frequency being that of NCOs, in favor of COs.[300]

Given that this CO assurance ensures a minimum effective DSB rate, what mechanism prevents excessive DSBs from forming? Once DSBs are formed in early prophase I, a third level of regulation is implemented, which is entirely dependent upon ATM kinase. SPO11–oligo complex levels are highly increased in *Atm* mutant mice, and *Atm* mutant cells are very sensitive to variations in SPO11 level, indicating that ATM acts upon DSB initiation to prevent further DSBs from forming, in a negative-feedback loop.[197,245,301]

CO products marked specifically by MLH1–MLH3 are also tightly regulated, in both their frequency and their distribution along chromosomes, in a process known as CO interference.[302] The phenomenon ensures that, once a CO occurs at one point along a chromosome pair, no other COs will take place nearby. If two or more COs occur along homologous chromosomes, they will be spaced far apart, to ensure that the stability of the bivalents is maintained during segregation. The precursors to Class I COs, MSH4–MSH5 foci, are also controlled by interference, indicating this is a step-wise process in mammals.[303] COs that are designated as Class II COs, mediated by MUS81–EME1, are not subject to interference, such that in species that only have the Class II system (e.g., *S. pombe*), interference does not play a role in CO placement.[304] In mice, the loss of *Mus81* results in a compensatory increase in the number of MLH1–MLH3 foci at pachynema, resulting in normal CO rates by diakinesis. Interestingly, these additional MLH1–MLH3 foci reduce the overall level of interference between foci, indicating that the additional foci may not follow interference rules.[278]

COs occurring on the pseudo-autosomal region of the XY chromosomes appear to have a different level of regulation from that of autosomal COs. DSBs occurring on the PAR are formed later than those on the autosomes, specifically by the alpha isoform of SPO11, and the chromatin of the pseudo-autosomal region appears to be packaged differently from the autosomal chromatin, indicating that the initiating DSBs may be under separate control.[201] Further evidence for this comes from the *Prdm9* mutant mouse, in which the DSBs are redistributed from recombination hotspots to promoter regions on the autosomes, but are formed normally on the PAR, indicating a PRDM9-independent mechanism for DSB formation in this region.[291]

Role of Small RNA Pathways in Mammalian Meiosis

A conserved feature of small RNAs is that they must be bound to an Argonaute protein for stability and/or function, with different classes of small RNA binding to distinct classes of Argonautes. The Argonaute protein family is divided into two clades. Those that exhibit greater homology to *Arabidopsis thaliana* AGO1 are the AGO-like proteins and include AGO1–4 in mouse and human (also known as EIF2C1–4), AGO1 and AGO2 in *D. melanogaster*, and Ago1 in *S. pombe*.[305] AGO-like proteins bind both small interfering RNA (siRNA) and micro-RNA (miRNA) species. The second clade of Argonaute proteins constitutes the PIWI-like clade, including proteins related to *D. melanogaster* PIWI, including *D. melanogaster* Aubergine (Aub) and AGO3, and mouse and human MILI, MIWI, and MIWI2.[305] *S. pombe* has no known proteins in the PIWI-like clade. PIWI-like proteins bind another class of small RNAs, known as piRNAs in mammalian systems.

AGO-like Proteins, siRNAs, and miRNAs in Gametogenesis

The AGO-like proteins rose to prominence due to their involvement in RNAi, an evolutionarily conserved mechanism of gene silencing whereby small RNAs are able to target mRNAs for degradation or translational inhibition. RNAi relies on the small RNA-binding capabilities and nuclease activity of an AGO-like protein within the RNA-induced silencing complex (RISC) to "slice" target mRNA. Slicing is achieved via the PIWI domain; however, only a subset of AGO-like proteins has this catalytic activity, despite the presence of the PIWI domain in all AGO-like proteins. The single *S. pombe* AGO1 protein has slicing activity,[306] along with two of the 10 *A. thaliana* AGO-like proteins, AGO1 and AGO4.[307,308,309] Of the four mouse–human AGO-like proteins, only AGO2 has slicing activity, despite the fact that all four mouse AGOs are able to bind small RNAs.[310,311] Recent studies have implicated mammalian AGO3 and AGO4 in regulating meiotic entry and in transcriptional silencing of the sex chromosomes during meiotic prophase I in males (discussed further in this chapter), while *Ago1* has nearly undetectable expression in germ cells.[69]

The biogenesis of miRNAs has been extensively reviewed[312,313] and involves transcription by RNA polymerase II to yield primary miRNAs, which are then cropped by the DROSHA–DGCR8 complex to yield shorter precursor miRNAs (pre-miRNAs). Pre-miRNAs consist of 60–70 nucleotide hairpin structures that are exported from the nucleus by exportin 5. Once in the cytoplasm, pre-miRNAs are processed by the RNase III nuclease DICER to remove the hairpin and to leave approximately 22-nucleotide miRNA–miRNA* duplexes. It is thought that the miRNA becomes loaded onto, and thus stabilized by, one of the four AGO proteins, while the miRNA* (or passenger) strand becomes degraded. Endogenous siRNAs (or endo-siRNAs) are produced in a similar fashion, except that they arise in situations where transcripts are able to form double-stranded RNA (dsRNA) or long stem–loop structures. These dsRNA transcripts are exported to the cytoplasm, where they, too, become the target for DICER and are then loaded onto AGO2.[313] Thus, a key distinction between miRNA and siRNA biosynthesis is the dependence (in the former) on DROSHA–DGCR8.

The relative roles of the siRNA and miRNA classes of small RNAs in germ cells have been discerned mostly through loss-of-function and conditional ablation of genes required for the generation and/or stability of these small RNA classes. For example, loss of *Dicer* by conditional deletion in the germline using Cre recombinase-mediated *LoxP* recombination has resulted in different phenotypes, depending on the *Cre* deletion mouse used and the lab from which the data were reported.[314] Since DICER is upstream of both the miRNA and siRNA pathways, it is not surprising that the most severe phenotype reported is that of complete testicular degeneration within days of birth in males, as a result of defects as early as the spermatogonial (premeiotic) stages when DICER is deleted from Sertoli cells.[315,316] *Dicer* deletion specifically in germ cells of male mice, in contrast, results in a variety of phenotypes, depending on the timing of CRE-mediated gene ablation. For example, loss of *Dicer* using a Neurogenin 3–*Cre* (*Ngn3–Cre*) mouse, which deletes around day 5 postpartum, results in slightly increased spermatocyte apoptosis but apparently normal meiotic progression for the majority of spermatocytes.[317,318] By contrast, deletion of *Dicer* during embryogenesis, using a *Ddx4–Cre* mouse, results in a more severe loss of spermatogenic cells and in infertility, resulting from delayed transition into, and through, early prophase I.[318] Two other groups utilized a *Stra8–Cre* strategy to delete *Dicer* prior to meiotic entry, and showed variable effects on meiotic progression, suggesting at least some role for DICER-driven events during prophase I in males.[319,320] Similarly, loss of *Dicer* in female germ cells results in meiosis I arrest and infertility.[321]

Loss of *Dgcr8* or *Drosha*, whose protein products play roles specifically in the miRNA pathway, also results in spermatogenesis defects from the spermatocyte stages onward,[322] while females remain fertile.[323] Loss of *Ago2*, which ablates siRNA function but not miRNA function, has no effect on germ cell proliferation or meiotic progression.[324] Taken together, these studies, while somewhat conflicting, point to a role for at least miRNA populations in meiotic progression. As will be discussed in this chapter, these functions may include regulating meiotic onset and/or transcriptional silencing.

Role of PIWI-Interacting RNAs in Mammalian Meiosis

The piRNAs were named for their exclusive association with Argonaute proteins of the PIWI clade, as opposed to the AGO clade that associates specifically with miRNA and siRNA species. PIWI clade proteins have been identified in a number of species, including *D. melanogaster*, for whose Piwi protein the clade was named. In mammals, three PIWI proteins exist—MILI, MIWI, and MIWI2—and the expression of these genes is restricted to germ cell lineages. Of the three major classes of small RNA found in mammals, piRNAs were discovered most recently and are highly enriched in germ cells. Importantly, while they were originally thought to be specific to germ cells, it now appears that they are found in other cells, including embryonic stem cells.[325] At 26–29 nucleotides in length, the piRNAs are larger in size than other small RNAs, and their biogenesis appears to be very different from that of other classes of noncoding RNA. Their synthesis is not dependent on DICER, and they arise from large genomic piRNA clusters that can exceed 100 kb in length and that encode many piRNAs of overlapping sequence.[326,327] Instead, their biogenesis appears to initiate by the formation of long single-stranded precursors transcribed from the piRNA loci, which are then processed by unknown mechanisms to form pre-piRNAs. These pre-piRNAs then load onto PIWI protein complexes for further processing and, in the case of pre-pachytene piRNAs, may be amplified through a *ping-pong* amplification mechanism, described in detail in Refs 313,327,328,329,330.

In mammals, piRNAs are most abundant during prophase I, but are also observed at high levels in prospermatogonia and, to a lesser extent, in oocytes.[328,331] Indeed, in male germ cells, two distinct populations have been identified: the pre-pachytene piRNA pool, which interacts with MIWI2 and MILI, and which is enriched in repeat-derived sequences,[328] and the pachytene piRNAs, which derive mostly from intergenic sequences and which interact with MILI and MIWI in pachytene spermatocytes and round spermatids.[332,333] Mouse knockouts for *Mili* and *Miwi2* result in spermatogonial stem cell loss, consistent with their interaction

with pre-pachytene piRNA populations. Conversely, deletion of *Miwi* in the mouse results in meiotic disruption, but spermatogenic failure does not occur until the round spermatid stage.[332,334,335,336,337,338]

The major function of pre-pachytene piRNAs, and their associated PIWI proteins, is to silence retrotransposons, through both transcriptional (epigenetic) and posttranscriptional (mRNA) activities.[335,339] This activity is distinct from the DNA methylation mechanism in which de novo methylation of retrotransposons occurs via the action of DNMT3L during fetal life.[340] Prior to meiotic entry, MILI and MIWI2 act to facilitate this de novo methylation such that the phenotype of *Mili* and *Miwi2* mutants mimics that of *Dnmt3l* loss, and MIWI2 appears to play a direct role in facilitating this methylation event.[327,337] Prior to prophase I, MILI may also act to cleave transposon mRNAs through its slicer activity.[327] MIWI, in contrast, appears to function exclusively during prophase I, associating with regulatory proteins that include the RNA-binding protein, mouse vasa homolog (MVH–DDX4), the ortholog of *Drosophila* Maelstrom (MAEL), and various Tudor domain-containing proteins, TDRD1, TDRD6, and TDRD7.[341,342,343,344] All of these proteins reside in the perinuclear diffuse structure known as the *Nuage* (meanining "cloud"), which is rich in RNAs and associated proteins. The mechanisms by which MIWI acts during pachynema remain unclear, but it appears to affect both mRNA stability and translational activity.[327] Part of the difficulty in understanding the role of MIWI during prophase I has been due to the fact that loss of *Miwi* (or any of the nuage proteins) does not deplete all the pachytene piRNAs in mouse spermatocytes, since MILI-associated piRNAs persist. Instead, conditional deletion of *Mov10l1*, a putative RNA helicase, during prophase I appears to selectively remove all pachytene piRNAs, and these mice exhibit massive DNA damage in postmeiotic round spermatids. These data indicate that pachytene piRNAs are required for maintaining genomic integrity postmeiotically, but that this function is not associated with any effects on retrotransposon repression.[345]

Meiotic Silencing

Meiotic silencing is the process by which unsynapsed chromatin is transcriptionally repressed during prophase I, and has been described in many organisms, including mouse, nematodes, fungi, and birds.[346,347,348] In the animal kingdom, meiotic silencing most commonly occurs in the heterogametic sex as a means of silencing the largely asynapsed sex chromosomes. This process has been termed meiotic sex chromosome inactivation (MSCI), and it is absolutely crucial for normal prophase I, since mouse mutants that fail to silence their sex chromosomes display meiotic arrest.[348,349,350,351] MSCI is just one example of a larger silencing mechanism called *meiotic silencing of unpaired chromatin* (MSUC), which can be directed at any region of the genome that fails to undergo synapsis (asynapsed). Thus, MSUC itself can be considered a pathological response to aberrant asynapsis, and usually results in apoptotic cell death, whereas MSCI is a normal feature of meiotic prophase I in male mammals and meiosis cannot proceed without it.

The purpose of meiotic silencing is not entirely proven, but it is thought to represent an ancient mechanism for suppressing heterologous chromatin as a means to protect the host genome from exogenous DNA, such as retrotransposons.[352,353] Alternatively, or in addition, meiotic silencing may serve to limit transcription from regions of damaged DNA,[354] or may prevent the expression of sex-linked genes that would otherwise be lethal to the process of prophase I (discussed further here). The existence of non-sex-chromosome-associated silencing mechanisms would argue against this latter option, however.

Meiotic Sex Chromosome Inactivation

During pachynema in mammalian spermatocytes, the X and Y chromosomes become sequestered into a defined heterochromatin-rich subdomain of the nucleus called the *sex body*. This domain is enriched in specialized histones,[246,355] proteins that mediate DNA damage responses through phosphorylation cascades,[348,356] and RNA.[69,357] Together, these mediate both sex body formation and silencing itself through mechanisms that are only slowly becoming elucidated. Histone marks associated with MSCI include H3K9me2,[358] ubiquitylated H2A,[352] and the phosphorylated histone variant of H2AX, γH2AX.[359] The importance of this latter modification is highlighted by the fact that mice lacking the critical phosphorylation site on H2AX fail to establish a sex body and cannot undergo prophase I, leading to infertility.[246,360] Interestingly, XO female mice, who possess only a single univalent X chromosome, also show sex body formation in a proportion of oocytes as a result of the failure to pair their single X.[351,352] As a result, these cells die because of silencing of essential X-linked genes without the presence of autosomal "backups". A proportion of cells exhibit self-synapsis of the X chromosome, which loops back on itself in a hairpin configuration, thereby protecting itself from the MSCI machinery and allowing the cells to proceed through meiosis.[348,351]

BRCA1 is an early marker of the sex body,[361] and this protein recruits ataxia telangiectasia and Rad-3 related (ATR) protein and its activator, TOPBP1.[197,356,362,363] These proteins assemble on the unsynapsed sex chromosomes in early pachynema, and they spread throughout the sex chromatin as pachynema progresses. This process of spreading is co-incident with ATR-mediated phosphorylation of H2AX, to become γH2AX, and

this mark becomes a defining feature of the sex body at pachynema.[246] Furthermore, the spreading of the BRCA1, ATR, and TOPBP1 signal throughout the chromatin may be mediated by MDC1, a binding partner of γH2AX, although their initial loading is not dependent on MDC1.[364] Importantly, the genesis of γH2AX at the sex body is temporally and functionally distinct from that of γH2AX found at sites of DSBs from leptonema to pachynema. Instead, it is likely that other kinases, perhaps ATM or DNA–PK,[365] are responsible for phosphorylation of H2AX and sites of SPO11-induced breaks.

The outcome of γH2AX appearance in the sex body is the condensation of the sex chromatin, and the exclusion of RNA polymerase II from the sex body, leading to transcriptional repression. The loss of expression from the X and Y is compensated for, in certain essential cases, by the expression of autosomal paralogs that are specifically active during male meiosis.[346] However, several sex-linked genes are known to be "toxic" during prophase I, and thus their expression must be repressed. These include the *Zfy1* and *Zfy2* paralogs, whose ectopic expression on the nonsilenced autosomes is sufficient to induce mid-pachytene apoptosis in transgenic animals.[366,367]

Analyses of meiotic mutants for genes involved in synapsis and/or DSB repair have indicated that the loss of these pathways also affects sex body formation and MSCI. Thus, spermatocytes with defective synaptonemal complexes or those that fail to properly repair their DSBs exhibit aberrant sex body formation, including those from homozygous mutant males for *Msh5*, *H2ax*, and *Sycp1*.[128,368] The loss of spermatocytes in these mutant animals occurs at mid-pachynema, when sex body silencing should be fully engaged, and is associated with increased expression of genes from the X and Y chromosomes.[368] However, it was unclear until recently whether loss of cells from these mutants was the consequence of checkpoint activation, due to persistent unrepaired breaks, or due to impaired MSCI. Royo et al. showed that in XYY males, in which single YY bivalents prevent transcriptional repression of Y-linked genes without any effects on DSB repair, the mid-pachytene loss was similar to that seen in DSB repair and synapsis mutants. Thus, it is likely that the loss of spermatocytes from many DSB repair mutant mice reflects improper Y-linked gene expression, and may account for the increased severity of the male meiotic phenotype compared to that of the female mutants for these genes.[350,368]

Meiotic Silencing of Unpaired Chromatin

As stated in this chapter, autosomes can also display meiotic silencing under certain conditions, and this is most clearly displayed in some of the mouse mutants for DSB repair and synapsis (some of which are listed here). This has led researchers to think more carefully about the links between synapsis, recombination, and the various silencing mechanisms. As described here, some of these meiotic mutants display synapsis defects that lead to normal MSUC, an example of this being *Spo11* homozygous mutant animals.[368] However, these mice exhibit disrupted MSCI.[198] Other mouse mutants, however, show aberrant MSUC as well as disrupted MSCI, and these include mice lacking *Msh5*, *Dmc1*, and *Sycp1*.[368] The interesting observation came with *Dmnt3l* homozygous mutant males, however, in which spermatocytes display increasing levels of asynapsis, and this is associated with a progressive decline in the efficacy of MSUC.[368] Importantly, the levels of MSUC-inducing BRCA1 associated with autosomal regions of asynapsis appear to be restricted such that it did not increase with the increasing severity of asynapsis in the *Dmnt3l* mutant spermatocytes, thus providing an explanation for failed MSUC. In addition, this BRCA1 relocation to the autosomes also results in reduced sex body-associated BRCA1, resulting in failed MSCI. Since BRCA1 initially localizes to DSB sites, this restricted pool of BRCA1 might also be sequestered at persistent DSBs in the face of impaired DSB repair pathways, perhaps providing an explanation for the impaired MSUC in DSB repair mutants (*Msh5*, *Dmc1*, etc.) but not in DSB-devoid mutants (*Spo11*).

Taken together, these different mouse mutants displaying extensive asynapsis with or without persistent unrepaired DSBs show differing MSUC responses, but all fail to silence their sex chromosomes.[368] This would suggest that the presence of asynapsed autosomes reduces the overall load of BRCA1 in the vicinity of the sex body, in turn reducing the recruitment of ATR. The amount of BRCA1 in females also appears to be somewhat limiting, since mouse oocytes bearing more than two or three asynapsed chromosomes fail to recruit enough BRCA1 to activate MSUC, whereas those with three or fewer asynapsed chromosomes possess enough BRCA1 to initiate silencing at each chromosome.[369]

One caveat to this model is the fact that some spermatocytes from *Brca1* mutant mice can evade meiotic arrest and might suggest an alternative mechanism for recruiting ATR. The obvious candidate for this alternative pathway is the 9-1-1 complex of RAD9, HUS1, and RAD1, which has been shown to accumulate in the sex body at pachynema,[354,370,491] and which is upstream of ATR in the somatic cell DNA damage repair pathway. Whether ATR is recruited to DSBs and the sex body by the same mediators is also the subject of debate at the current time.

Small RNA Participation in Meiotic Silencing

The involvement of small RNAs in meiotic silencing was first alluded to in *Neurospora crassa*, where the associated process of MSUD was first shown to involve Sad1, an RNA-directed RNA polymerase.[371] In

mammals, recent studies have demonstrated that the small RNA–associated Argonaute protein AGO4 is localized to the sex body during pachynema and to regions of induced asynapsis (e.g., in translocation mutant mice) during prophase I.[69,372] This is an exciting finding because mammalian Argonautes commonly perform their small RNA functions exclusively in the cytoplasm and are not known to reside in the nucleus of somatic cells, unlike the situation in worms and other organisms where nuclear Argonaute complexes have been functionally characterized.[312,373] In mouse spermatocytes, however, AGO4 and, to a lesser extent, AGO3 both reside in the sex body, and are associated with many miRNAs that originate from both the sex chromosomes and the autosomes.[69] Loss of *Ago4* results in altered sex body morphology and failure to silence many genes on the X and Y chromosomes, including *Zfy1* and *Zfy2*, whose expression is toxic to the cells. However, AGO3 can partially compensate for loss of AGO4 such that *Ago4* mutant mice are only subfertile. Importantly, loss of *Ago4* results in dramatic downregulation of miRNAs from pachytene spermatocytes, over half of which originate from the sex chromosomes.[69] This is counterintuitive to the finding that RNA polymerase II access to the sex body is upregulated in these animals, but fits well with other studies demonstrating that specific sex-linked small RNAs evade the normal silencing mechanism that is characteristic of the sex chromatin during pachynema.[374] Moreover, these data fit with those from *Dicer* conditional knockout mice showing that deletion of *Dicer* (which is required for miRNA and siRNA pathways upstream of AGOs) results in failed MSCI.[320] Taken together, these observations suggest that small RNAs, and their associated AGO proteins, play essential roles in mediating the silencing of unpaired chromatin during meiotic prophase I in mammals in a mechanism that may resemble the process of RNA-induced transcriptional silencing found in *S. pombe*.[375,376] How these RNA-driven processes interface with the known molecular pathway that induces meiotic silencing is unclear at the present time, and is the subject of intense investigation.

Checkpoint Control of Synapsis and Recombination

There are many processes active during meiosis that ensure that the correct number of gametes arises and that defective gametes are eliminated from the population. Without these safeguards in place, defective gametes may well be utilized for reproduction, resulting in the inheritance of mutations in the subsequent generation. In prophase I, there are two important surveillance mechanisms, or checkpoints, that monitor the developing gamete for any abnormalities, namely, the synapsis checkpoint and the recombination checkpoint. These two checkpoints monitor nuclear events, ensuring that complete synapsis between homologs and effective repair of DSB intermediates, respectively, have been completed before the cell can progress beyond prophase I. So, for example, spermatocytes lacking *Spo11*, which fail to undergo complete synapsis of their chromosomes but which have no DSBs and no recombination defects, succumb to the synapsis checkpoint,[198,199] while mutation of mouse *Trip13*, the ortholog of yeast pachytene checkpoint 2 (PCH2), results in loss of prophase I cells at pachynema without affecting synapsis but instead resulting in recombination failure at a subset of DSB events.[377] The apoptosis of cells at the pachytene checkpoint in *Trip13* mutants is thus intact, despite normal synapsis (the opposite situation to that seen for *Spo11* mutant mice), providing evidence that failed recombination alone can trigger this checkpoint.

Other mutants that affect DSB repair (e.g., *Msh4*, *Msh5*, and *Dmc1* mutants) also succumb to the recombination checkpoint.[349,378] However, given that defects in DSB repair often result in synapsis failure too, it is often difficult to separate the two checkpoints from each other, and thus, they are collectively termed the pachytene checkpoint. Importantly, most of these mutants also exhibit failures in MSCI, while failure to induce MSCI itself results in activation of the pachytene checkpoint, leading to the proposal that failed MSCI may comprise a major mechanism for checkpoint induction in mammalian meiosis.[349]

In males, this checkpoint is extremely stringent and serves to eliminate defective cells at pachynema via the process of apoptosis. Indeed, even in wild-type male mice, there is an abundance of apoptotic cells present in the testes, indicating that the checkpoint is highly efficient in wild-type male mice.[379] In females, the checkpoint appears to be much less rigid, such that gametes bearing synapsis and recombination defects may progress beyond pachynema. Oocytes with more minor abnormalities can also progress to the end of meiosis, perhaps maintaining these errors from prophase I in the form of aneuploidies in the resulting gamete.[380] This sexual dimorphism is largely poorly understood in mammals; however, the general thinking is that because males make millions of sperm throughout their lifetime, whereas females only make oocytes once prior to birth, the amount of effort invested by the female organism in generating the gamete is such that they simply "make do" and repair their defects as best they can, whereas males eliminate these defective cells entirely.

It is important to note that defects later in prophase I, after synapsis and recombination are largely completed, may also result in apoptosis, but that this checkpoint may not occur until metaphase I in males, and even later in females. One example of this is *Mlh1*-deficient mice. *Mlh1* mutant males exhibit almost complete loss of chiasmata in diakinesis, with cells undergoing apoptosis

around the time of metaphase.[270] *Mlh1* mutant oocytes progress somewhat further, however, with a proportion of them extruding a first polar body, albeit at low rates and with highly likelihood of nondisjunction.[381,382] Similar results are observed in *Mlh3* mutants,[272] and in both cases are associated with poor bipolar attachment on the first meiotic spindle and subsequent defects in spindle morphology. These data suggest that later prophase I defects evade the pachytene checkpoint, but instead are identified by a second checkpoint upon attachment to the meiotic spindle.

CHROMOSOME SEGREGATION AT METAPHASE I (MI) AND MEIOTIC PROGRESSION

A fundamental and defining feature of the mitotic and meiotic divisions is the ability to sequentially and equally segregate genetic material to opposite poles and thus to produce daughter cells that contain the same amount of genetic material through two rounds of division, one equational and one reductional (Figure 1.1). During mitosis or during the second meiotic division, this segregation involves sister chromatids that are, by necessity, held together by cohesion (discussed further here) until they become captured by microtubules emanating from each mitotic spindle pole (Figure 1.12). Sister chromatid cohesion is an essential prerequisite for their attachment to microtubules emanating from opposite poles, a configuration known as amphitelic attachment (or bi-orientation; Figure 1.13), which, in turn, ensures the equal segregation of sister chromatids at anaphase. By contrast, during the first meiotic division, segregation separates the homologous chromosomes, each of which consists of paired sister chromatids (Figure 1.1). The role of prophase I events is to maintain homologous chromosome interactions up until the time of segregation. Thus, by the end of prophase I and into metaphase I, chiasmata-intact chromosomes align along the midplate of the cell and initiate the first meiotic division. This process is tightly regulated to ensure that homologous chromosome pairs align appropriately along the midplate in the correct bi-orientation to facilitate segregation of homologs to opposite poles of the cell, while sister chromatids remain mono-oriented (unlike during mitosis), or in syntelic attachment, to ensure that they move toward the same pole (Figure 1.13). Thus, a fundamental distinction between the two meiotic divisions is the syntelic versus amphitelic attachment behavior of the sister chromatids.

In order for these events to occur normally, chromosomes must engage a functional bipolar spindle and must also stabilize the spindle to ensure appropriate tension with which to facilitate equal segregation. Destabilization of the spindle can result in loss of tension, which will increase the potential for one or more chromosomes to fail in their attempts to move to the appropriate pole. One of the key processes that prevents catastrophe at this critical time is the spindle assembly checkpoint (SAC), a system that detects imbalances in the meiotic spindle (caused by loss of tension and/or unstable chromosome connections) and induces metaphase arrest until the problem is resolved. Many of the components of the SAC are preserved between mitosis and meiosis; however, by necessity, their functions extend in meiosis to ensure that homologous chromosomes, rather than sister chromosomes, become stably bi-oriented and attach to the spindle.

Our ability to study meiotic spindle assembly and checkpoint control has been limited largely to studies in oocytes, since disruption of spindle structures in male gametes using microtubule-destabilizing drugs results in early prophase I defects and arrest, precluding analysis of metaphase I and beyond.[383,384] Thus, much of what will be discussed in this section will focus on studies performed in lower eukaryotes and in females, with extrapolation where possible to analogous events in males. The relative difficulties in monitoring spindle assembly and progression in males are just the tip of the iceberg when it comes to differences in meiotic progression between the sexes. Indeed, female meiosis imposes several complexities on the process of spindle assembly and stability, not least of which is the long length of time during which the chromosome chiasmata must persist from their establishment in utero during prophase I to their critical role on the meiosis I spindle following meiotic resumption in the adult. Indeed, the entire spindle stability rests on the integrity and robustness of the chiasmata, the *raison d'être* for prophase I events in the first place. The importance of proper chromosome segregation during meiosis cannot be overstated, since chromosome mis-segregation during the first meiotic division can result in the generation of gametes containing the wrong number of chromosomes, a condition known as aneuploidy. Aneuploidy most commonly arises due to mis-segregation of homologous chromosomes, referred to as nondisjunction, but also can arise during the second meiotic division. As will be discussed in this section, nondisjunction is particularly prevalent in humans and, more specifically, in females.

The Spindle Assembly Checkpoint

The SAC is conserved between mitosis and meiosis, and is essential for monitoring the accurate segregation of genetic material, thereby signaling to the cell cycle machinery to continue the cell cycle or to arrest the cell in metaphase. This system acts at the level of the spindle to ensure that all chromosomes are attached to microtubules emanating from the appropriate pole, and to

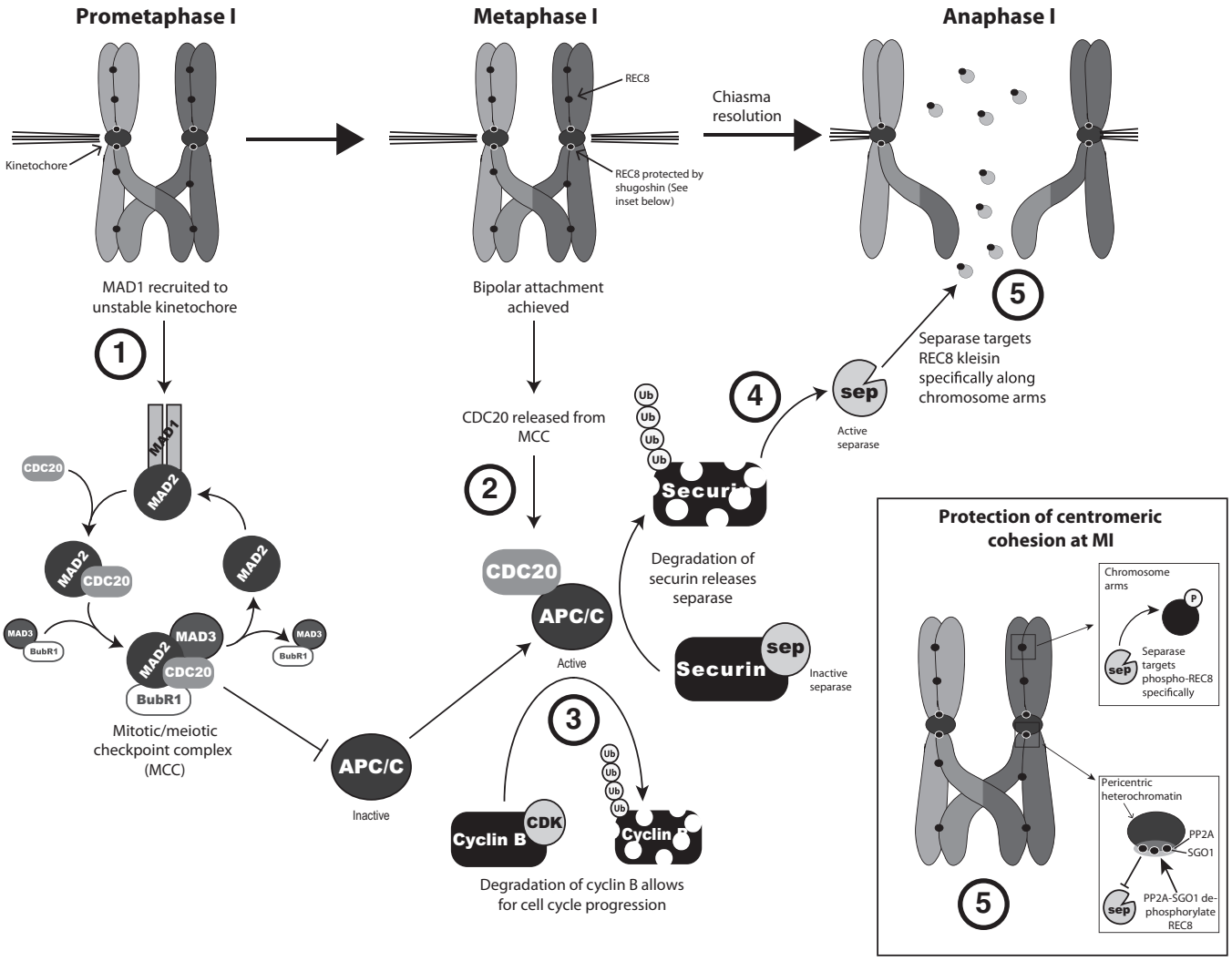

FIGURE 1.12 Summary of events at the metaphase-to-anaphase transition in mammalian meiosis. Step 1: Spindle assembly checkpoint (SAC) components are recruited to unstable kinetochores through the initial association of MAD1 and the subsequent recruitment of the mitotic–meiotic checkpoint complex (MCC). This inhibits APC/C activation. Step 2: If and when bipolar attachment is achieved, the MCC is destabilized, releasing CDC20, which can then bind to, and activate, APC/C. Step 3: APC/C can then ubiquitylate cyclin B–CDK complexes, resulting in their destruction, which facilitates cell cycle progression. Step 4: At the same time, APC/C ubiquitylates securin, releasing separase, which can then mediate phospho-REC8 destruction along chromosome arms, a process that is essential for disjunction of homologous chromosomes. Step 5 (inset panel): Importantly, SGO1 together with PP2A dephosphorylate REC8 at the centromere, which prevents separase-mediated destruction of REC8 at this location and thus preserves sister chromatid cohesion.

monitor tension within the spindle. Once all of these processes are in place, the SAC is silenced, and the anaphase-promoting complex/centrosome (APC/C) is activated, resulting in chromosome or chromatid movement to opposite poles and anaphase entry.

The SAC was discovered through a series of genetic screens in budding yeast that led to the identification of the key components of the checkpoint: the mitotic arrest deficient proteins, including Mad1, Mad2, and Mad3 (BUBR1 in vertebrates); and the budding uninhibited by benzimidazole proteins, Bub1 and Bub3, along with Mps1.[385,386,387,388,389,390,391] Classic laser ablation studies demonstrated that unattached kinetochores generate an inhibitory signal that delays anaphase onset, while destruction of the centromere (including both associated sister kinetochores) results in release of the checkpoint and anaphase progression. These studies indicated that activities in the vicinity of the kinetochore are responsible for monitoring mono-orientation and kinetochore attachment and for facilitating metaphase-to-anaphase progression.[392,393] Accordingly, most SAC components were later localized to the kinetochore,[387] and were found to be highly conserved in vertebrates.[394,395,396,397,398,399]

BUB1 is a protein kinase that localizes to kinetochores, senses microtubule attachment and stability, and may be involved in regulation of cyclin B (Figure 1.12, step 1). Similarly, MAD1 is recruited specifically to unattached, mono-oriented

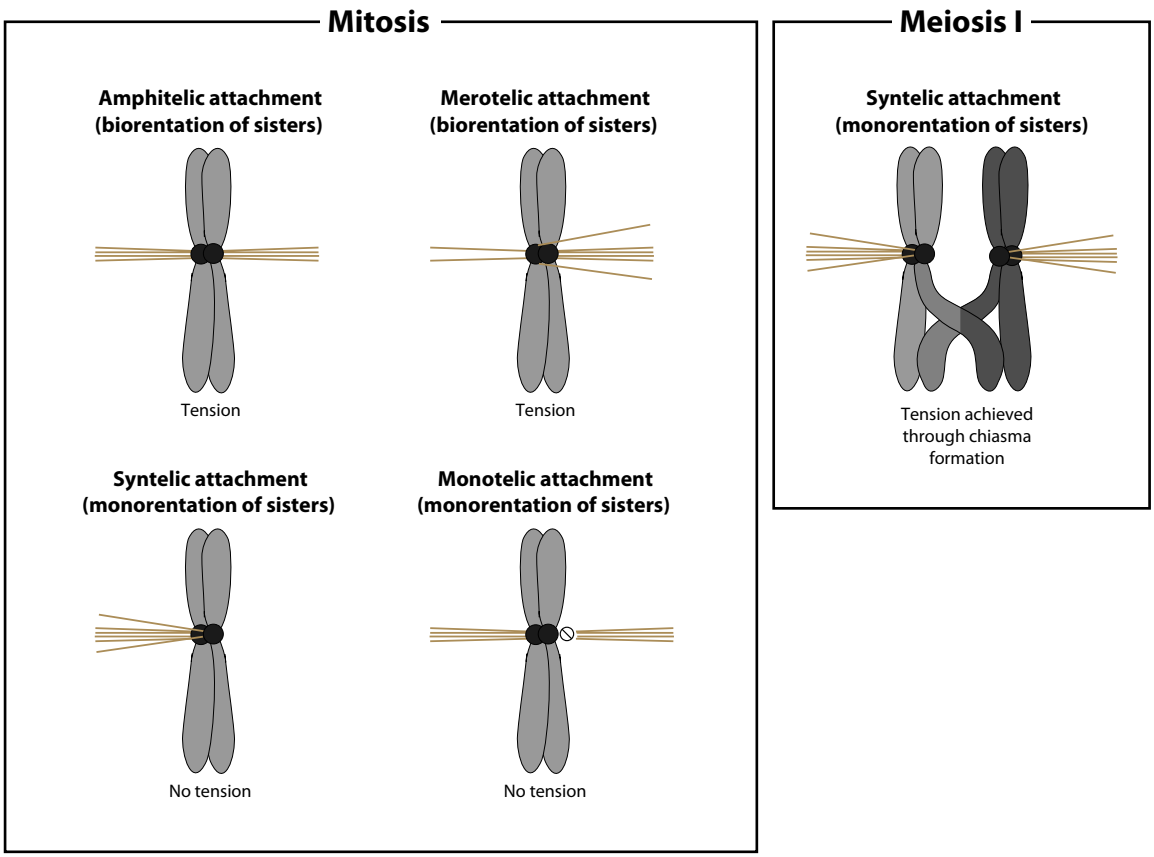

FIGURE 1.13 Sister chromatid interactions with spindle microtubules during mitosis and meiosis. During mitosis, sister chromatid kinetochores must attach to microtubules in bi-orientation to ensure tension across the spindle and, thus, equal segregation at metaphase. This attachment is referred to as amphitelic. However, bi-orientation can be achieved through merotelic attachment, although these interactions can be somewhat less stable. Mono-oriented attachment (such as those shown in the left-hand box) result in loss of spindle tension. During meiosis I (right-hand box), sister kinetochores must be mono-oriented toward the same pole, resulting in syntelic attachment. Tension across the metaphase I spindle is achieved through stable chiasmata formation.

kinetochores, and recruits with it MAD2. The localization of MAD1–MAD2 complexes at centromeres is at least indirectly dependent on MPS1.[400,401,402,403] MAD2 is capable of heterodimerizing and thereby recruits free MAD2 from the cytosol.[387] Upon spindle damage in mitotic cells or in the presence of unattached kinetochores, BUB1 phosphorylates MAD1, leading to the dissociation of MAD1–MAD2 complexes from the kinetochore. This dissociation allows MAD2 to sequester CDC20, an activator of APC/C. The formation of complexes of MAD2 and CDC20, together with BUBR1 and BUB3 (collectively known as the mitotic–meiotic checkpoint complex, or MCC; Figure 1.12, step 1),[387] prevents activation of APC/C and thus prevents the ability of the latter to invoke anaphase progression. Loss of this SAC mechanism in mammalian cells causes premature sister chromatid disjunction, leading to mis-segregation of chromosomes and cell death.[393,404,405]

During meiosis, the SAC acts at both metaphase I and II, and the functional components of the SAC appear to be conserved through both divisions.[403] During meiosis I, however, bi-orientation of sister kinetochores is prevented and instead the tension necessary to ensure stable microtubule attachment is provided by the chiasmata, which maintain homolog interactions (Figure 1.13). Moreover, during meiosis in the mouse, the preferred activator of the APC/C is CDH1, rather than CDC20, which is utilized during mitosis (Figure 1.12, step 2).[406] Studies of XO mice have demonstrated that, despite the absence of a pairing partner for the single X chromosome, failure to pair a single chromosome in oocytes does not result in meiotic failure, although the females exhibit somewhat reduced fertility. This has led to suggestions that the SAC is absent in vertebrate oocytes.[407,408] This does not appear to be the case, however, since overexpression of dominant negative forms of *Bub1* in mouse oocytes results in accelerated completion of MI and polar body extrusion (PBE).[409] Moreover, conditional mutant mice that do not express *Bub1* specifically in their oocytes display massive chromosome mis-segregation at meiosis I, precocious loss of cohesion between sister chromatids, and premature resolution of chiasmata and PBE.[410] These accelerated events

are the result of early activation of APC/C.[410] BUB1 appears to have additional roles within the context of the meiosis I metaphase-to-anaphase transition, namely, it is required for congression of bivalents onto the metaphase I spindle, and to prevent premature destruction of sister–centromere cohesion (discussed further here). Interestingly, loss of one allele of *Bub1* in oocytes causes similar meiotic defects to homozygous deletion at this locus, whereas loss of a single copy of *Bub1* in spermatocytes has no such effect,[411] perhaps due to the existence of alternative testis-specific *Bub1* transcripts.[412]

In line with the differences in sister chromatid orientation during the first meiotic division, MAD2 localization during meiosis I in mouse spermatocytes differs from that seen during mitosis. MAD2 is present at most, if not all, kinetochores from prometaphase of the first meiotic division, and remains associated with the kinetochore throughout MI. It does not dissociate during metaphase and anaphase, as is the case for mitosis. Thus, progression to anaphase at the first meiotic division is not dependent on loss of the SAC complexes from kinetochores, at least in male mammals. In contrast, MAD2 localization during MII mimics that seen in mitotic cells, initially localizing to most kinetochores at the start of the second meiotic division, and then being lost as chromosomes arrive at the spindle equator.[413] Thus, loss of MAD2 from MII kinetochores during male meiosis occurs once stable spindle connections have been established and, presumably, facilitates the release of MAD2-mediated APC/C inhibition.

The aforementioned SAC proteins all function at the kinetochore, with the exception of MAD1 in mouse oocytes, which assembles at the spindle pole and from there is recruited to the kinetochore when unstable microtubular connections are formed.[414] By contrast, MAD1 in *Xenopus* oocytes remains kinetochore associated and is responsible for recruitment of MAD2 and other proteins to unattached kinetochores.[398] Regardless of its position on the spindle, MAD1 is thought to act as a sensor of spindle tension, whereby loss of tension induces and reinforces MAD1 localization at the kinetochore, which, in turn, inhibits APC/C activity.[403] This localization is dependent on preloading of MPS1, whose activity also appears to be required for meiosis in a number of organisms, including yeast, flies, mice, and zebrafish,[415,416,417] and this involvement extends to both meiotic divisions.[402] MPS1 activity may also be required for cytostatic factor (CSF) arrest at metaphase II (as discussed further in this chapter; and see Ref. 418).

The First Meiotic Division: Kinetochore Orientation and Cohesion Function

Given their similar progression and molecular regulators, the processes of mitosis and meiosis are often compared, particularly with respect to spindle assembly and chromosome segregation. The most obvious differences, however, are observed at the first meiotic division when, by the end of meiotic prophase I, when the synaptonemal complex breaks down, bivalent homologous chromosomes are seen to be connected by their chiasmata. The homologs each consist of paired sister chromatids, held together through cohesion, while cohesion also serves to maintain homolog interactions distal to the chiasmata sites. This arm cohesion must be lost during the first meiotic division, while maintaining cohesion at the centromeres to ensure that sister chromatid pairing remains intact through meiosis II (Figure 1.12, step 5). Meiosis-specific centromeric cohesion is also important as it serves to facilitate differences in the way that the centromeres attach to microtubules during the first meiotic division (Figure 1.12, step 5). In stark contrast to the events in mitosis, and in order to ensure spindle tension, the homologous sister chromatid pairs (i.e., two chromatids) must attach to microtubules emanating from the same pole such that their microtubule attachment points at the centromere (large protein complexes known as kinetochores) are mono-oriented toward one pole, while the opposing homologous sister chromatid pair is mono-oriented in the opposite direction (Figure 1.13). Indeed, in yeast, amphitelic attachment of sister chromatids is actively prevented by the monopolin proteins.[419] To this end, sister kinetochores are fused or closely aligned so as to ensure their syntelic attachment to microtubules from a single spindle pole, while the fused kinetochores from the opposing homolog (or bivalent) are captured in syntelic fashion by microtubules from the opposing spindle pole, to achieve bipolar tension and stability of the overall spindle.

Metaphase-to-Anaphase Transition during the First Meiotic Division

The downstream target of the SAC is the anaphase-promoting complex, or cyclosome (APC/C), a multimeric ubiquitin E3 ligase whose activity targets several proteins for degradation.[420] APC/C is activated by CDC20 or by CDH1, and the association of these activators with APC/C is downregulated by sequestration of CDC20–CDH1 by MAD2 when the SAC is activated.[406] Thus, when chromosomes align correctly and spindle stability is ensured, the release of CDC20 or CDH1 from the MAD2 complex allows for activation of APC/C (Figure 1.12, step 2). APC/C then targets a number of key proteins for degradation via ubiquitylation, and these include securin and cyclin B (Figure 1.12, steps 3 and 4). Securin is an inhibitory chaperone of the protease separase. During mitosis, separase cleaves the α-kleisin RAD21, resulting in the uniform loss of cohesion across the paired sister chromatids, and leading to chromatid movement to opposite spindle poles.[406]

Studies in yeast, flies, and vertebrates have indicated that the APC/C–CDC20–CHD1 system is also responsible for metaphase–anaphase progression during meiosis and that securin is the major target of APC/C.[421,422] Events during meiotic prophase I are somewhat different with respect to cohesion, with the meiosis-specific α-kleisin, REC8, being used in place of RAD21. At the end of prophase I, with the synaptonemal complex degraded, cohesins distal to the chiasmata maintain cohesion along the chromosome arms and provide the tension and pulling force necessary for correct bivalent alignment on the metaphase I spindle. Once stable chromosome alignment is achieved, APC/C is activated, resulting in the degradation of securin and cyclin B, and this leads to cleavage of REC8 by active separase, thus inducing anaphase I onset in a manner analogous to that seen in mitosis. Importantly, this cleavage event occurs in concert with resolution of the chiasmata and only across the chromosome arms, allowing homologs to move to opposite poles, while retaining sister chromatid attachments' pericentromeric region (Figure 1.12, step 5).[423] The mechanism by which this differential protection of cohesion is achieved was uncovered in three distinct yeast screens for mutants affecting Rec8-mediated cohesion.[424,425,426] In the first, Kitajima et al. demonstrated that replacement of Rad21 by Rec8 during mitosis in fission yeast was not sufficient to replicate the stepwise loss of cohesion at anaphase, indicating that it was not Rec8 itself that promoted this centromeric protection phenomenon.[424] Furthermore, ectopic expression of a cleavage-resistant *Rec8* prevented sister chromatid separation, suggesting that, unlike in meiosis, mitotic separase is capable of cleaving Rec8 in a timely fashion. Thus, the existence of a meiotic-specific "centromeric protector of Rec8" was postulated, and a suitable candidate emerged in one screen for mutants that are toxic when expressed mitotically in the presence of Rec8, as well as in other screens for mutants that exhibit abnormal chromosome segregation during meiosis.[424,425,426] One gene was identified and named *Shugoshin*, a Japanese word meaning "guardian spirit", the budding yeast homolog for which was named *Sgo1*. Further homology searches revealed a second paralog in fission yeast, *Sgo2*, as well as orthologs in many species, including *Drosophila MeiS322*, which had been previously described as a gene essential for centromeric cohesion.[423,427,428]

As expected, both Sgo1 and its paralog, Sgo2, are meiosis specific, and both localize to centromeres in a Bub1-dependent fashion.[423,424,429] Importantly, their distribution alters radically in meiosis II, with SGO2 redistribution throughout the centromere facilitating separase-induced cohesin cleavage at this site.[430] During meiosis I, however, Shugoshin proteins prevent removal of cohesins from the centromere, at least in part by recruiting protein phosphatase PP2A to the pericentromeric region. PP2A is a serine–threonine phosphatase that, together with Shugoshin, can dephosphorylate the usually heavily phosphorylated Rec8 (Figure 1.12, step 5, inset).[431,432] In mice and in yeast, localization of PP2A to the centromere is dependent on Shugoshin,[433] while loss of PP2A in yeast results in premature loss of centromeric cohesion.[431,434]

While loss of Rec8 phosphorylation is now established as an important feature of maintenance of centromeric cohesion, the kinases involved in initial hyperphosphorylation remain the subject of debate. Clearly, Rec8 is heavily phosphorylated under normal conditions, and this phosphorylation is essential for cleavage by separase. In yeast, three kinases have been implicated in Rec8 phosphorylation, namely, polo-like kinase (Cdc5), casein kinase 1δ/ε (CK1), and Dbf4-dependent Cdc7 kinase (DDK)[423,435] and a number of reports present conflicting data with regard to the relative importance of each of these kinases. On the one hand, loss of Cdc5 in budding yeast during meiosis results in hypophosphorylated Rec8 and a failure to cleave cohesin,[436] while during mitosis in yeast, cohesion cleavage is promoted through phosphorylation of the cohesin subunit, Scc1, by Cdc5.[437] On the other hand, mutation of the Cdc5 target sites on Rec8 has surprisingly little effect on cohesin cleavage in meiosis I,[438] while, under certain mutant conditions, Rec8 is cleaved even when *Cdc5* is not expressed.[439,440] Thus, it is more likely that Cdc5 may act further upstream of Rec8 to modulate cleavage, although not directly on Rec8 itself. Instead, it appears that CK1 and DDK may instead be the kinases directly responsible for Rec8 phosphorylation since combined deletion of these kinases results in a block in Rec8 cleavage.[435] It is also possible that other kinases may participate in these events, since deletion of CK1, for example, does not cause as severe a block to Rec8 cleavage as does mutation on the CK1 phosphorylation sites on Rec8.[441]

Resumption of Meiosis in Females following Dictyate Arrest: The Roles of MOS, MPF, and CSF

A key feature of meiotic progression in female vertebrates is the punctuated progression of meiotic events. In mammals, prophase I progresses through fetal life up until the point that chromosomes are fully synapsed and COs are evident. This is the stage of diplonema, and the synaptonemal complex has, by this time, broken down. The process of prophase I arrests at this point, in a stage known as dictyotene or dictyate arrest. In many animals, this occurs at around the time of birth, and may be semisynchronous across all the oocytes in an individual.

At this stage, the oocytes are in a structure known as a cyst in which many common cytoplasmic components

are shared. The role of the cyst is unknown, but one common belief is that the cyst structure allows for one or a few oocytes to gain nutritional and energy advantage, while the others die off, perhaps explaining the huge wave of oocyte death, or atresia, at birth. During dictyate arrest, the oocyte grows in size somewhat, and the pre-granulosa cells form primordial follicles, thus dissolving the cyst structure. The follicles wait in this state until the correct endocrine cues to resume division, or activation.

Meiosis does not resume in mammals until after puberty, at which time circulating gonadotrophins induce ovulation and meiotic resumption and completion of the first meiotic division. The major factor that induces meiotic resumption is the maturation promoting factor (MPF), so named in 1971 by Masui and Markert.[442,443] While its effects in oocytes had been known for several decades, it was not until the late 1980s that the constituents of MPF became known: cyclin B and CDK1.[444,445]

MPF activation is dependent on at least three activities of luteinizing hormone (LH; Figure 1.14(A)). Prior to the surge in LH at estrus, and in order to maintain dictyate arrest, MPF activation is prevented by high levels of cyclic adenosine monophosphate (cAMP) in the oocyte. cAMP levels are kept high by both intrinsic and extrinsic factors. In the case of the latter, cAMP is provided by the surrounding granulosa cells via gap junctions that pervade the zona pellucida, the glycoprotein coat surrounding the oocyte. These gap junctions also supply cyclic guanosine monophosphate (cGMP), which suppresses oocyte phosphodiesterase 3A (PDE3A), which would otherwise degrade cAMP (reviewed by Ref. 446). Elevated cAMP results in increased protein kinase A (PKA) levels that phosphorylate key kinases, WEE1 and MYT1. These kinases, in turn, phosphorylate MPF. Phosphorylated MPF is inactive and cannot induce meiotic resumption. PKA also phosphorylates (and inactivates) CDC25B, the major phosphatase required to activate MPF.

Upon LH stimulation, cAMP levels decline due to an effect of LH on the granulosa: First, LH induces a mitogen-activated protein kinase (MAPK) cascade that results in reduced cGMP levels, which facilitates increased PDE3A hydrolyzing activity to degrade cAMP in the oocyte. Next LH reduces cAMP in the oocyte by directly upregulating PDE3A hydrolysis of cAMP. Third, LH induces mitochondrial sequestration of PKA by A-kinase anchoring proteins (AKAP) and facilitates the dephosphorylation of CDC25B, which can then remove the phosphate group from CDK1 and thus activate MPF.

A key step in the activation of MPF, at least in females, involves MOS, the product of the Maloney murine sarcoma virus *c-Mos* proto-oncogene. MOS is a protein kinase that functions upstream of the MAPK cascade that ultimately results in the phosphorylation and activation of $p90^{RSK}$. MOS and $p90^{RSK}$ downregulate MYT1–WEE1, thus promoting further the activation of MPF and progression through to anaphase I.[447]

Induction of metaphase I results in completion of the first meiotic division, which involves condensation of the chromosomes, assembly onto the meiotic spindle, and nuclear envelope breakdown, also called germinal vesicle breakdown (GVBD). Instead of cleaving into two daughter cells, however, the excess half complement of chromosomes are shunted into a rudimentary structure called the first polar body (PB1 in Figure 1.14) in a clearly asymmetric division.

There is no intervening S-phase before entry into meiosis II, and oocytes progress to metaphase II rapidly, only to arrest again under the influence of cytostatic factor (CSF; Figure 1.14(B)). High CSF activity induces stabilization and reaccumulation of MPF again by inhibiting the action of the anaphase-promoting complex, APC/C, which would otherwise target MPF for proteosomal degregradation via polyubiquitination of cyclin B[406] (Figure 1.14(C)). MOS is also important for maintenance of high levels of MPF,[448] possibly upstream of CSF, in turn resulting in the second meiotic arrest at metaphase II (MII). It is only upon fertilization that this block is removed, via the influx of calcium ions that is triggered by sperm penetration, allowing MII progression once the sperm enters the egg.[449] Thus, if eggs remain unfertilized, the MII arrest persists. In contrast, *c-Mos* knockout female mice exhibit parthenogenetic activation of unfertilized oocytes, indicating that loss of *c-Mos* allows oocytes to proceed to the second meiotic division without the need for fertilization.[450,451] Importantly, MOS does not appear to be required for meiotic progression in male mice.

Despite its initial characterization in the 1970s, along with MPF, the molecular nature of CSF has never been completely resolved. It is plausible, given the relationship between MPF and APC/C activity, that CSF is an inhibitor of the latter and/or may constitute one or more components of the MCC (Figure 1.13). However, loss of one or more components of the MCC in oocytes does not affect APC/C function, nor does it affect calcium-induced loss of CSF activity,[452] suggesting that SAC proteins do not constitute the elusive CSF. Instead, it has been suggested that MOS–MAPK constitutes the true CSF activity, since introduction of *Mos* into a blastomere of a two-cell embryo results in CSF-like arrest, while both MOS protein and mRNA are rapidly degraded upon fertilization.[452] However, the mechanism by which the downstream target of MOS, $p90^{RSK}$, acts to suppress APC/C activity remained elusive for many years. Recent studies have pointed to EMI2 as a mediator of

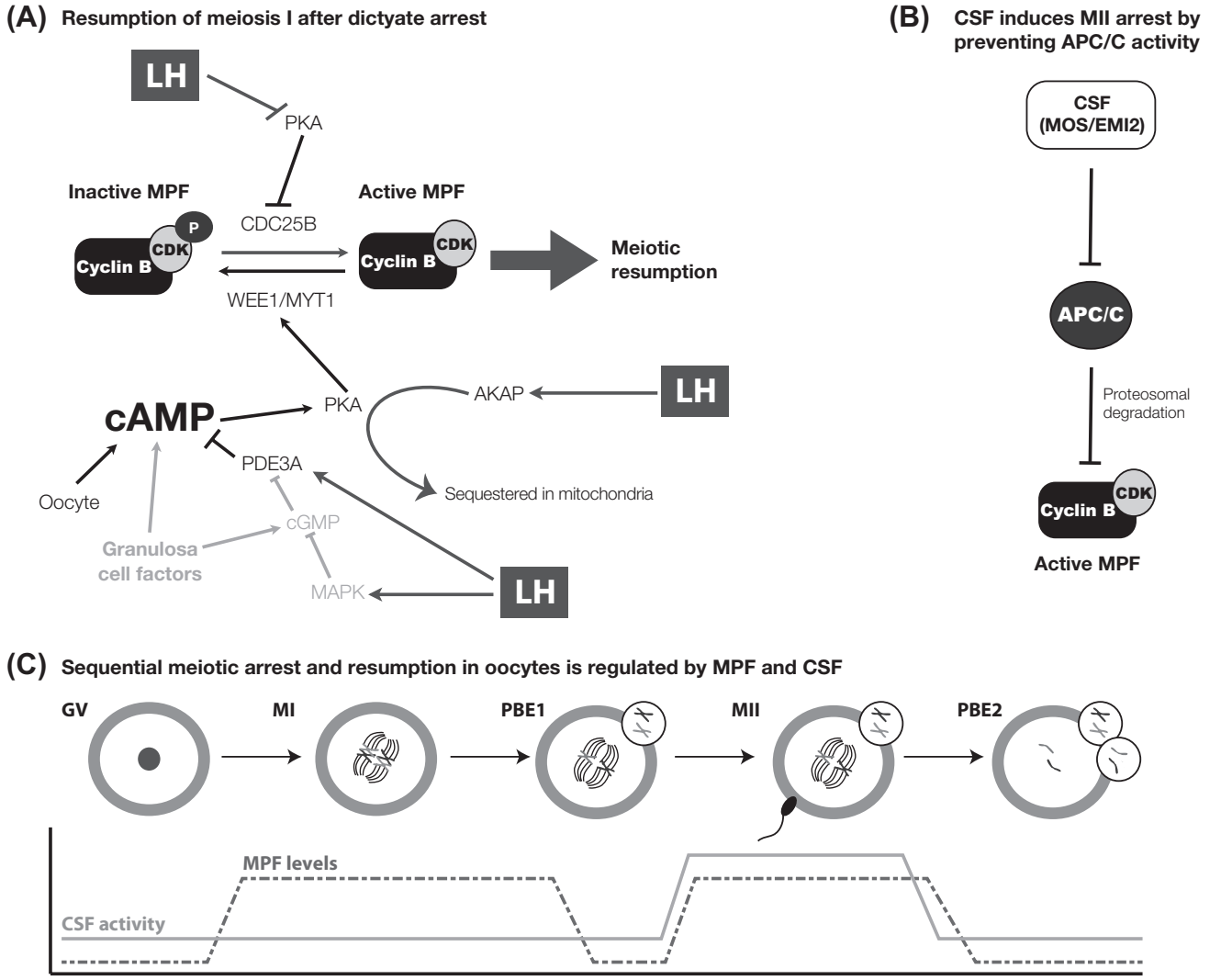

FIGURE 1.14 Resumption of meiosis I after dictyate arrest in females is mediated by MPF and CSF activity. (A) Dictyate arrest is achieved through the inactivation of MPF (cyclin B–CDK) via phosphorylation of CDK. This phosphorylation is brought about by maintaining high levels of cAMP within the oocyte. cAMP activates protein kinase A (PKA) that phosphorylates WEE1–MYT1 to maintain the phosphorylation status of CDK. PKA also inhibits CDC25B, the phosphatase that would ordinarily dephosphorylate cyclin B–CDK. Upon stimulation by luteinizing hormone (LH), PKA is inhibited through several routes, first by increasing PDE3A-mediated hydrolysis of cAMP through several routes. LH induces the MAPK cascade within the supporting granulosa cells that possess LH receptors, and this, in turn, suppresses cGMP release. In the dictyate-arrested oocyte, cGMP usually passes through gap junctions into the oocyte, where it suppresses hydrolysis of cAMP by PDE3A, and thus suppression of cGMP in the granulosa cells overcomes this suppression. In addition, LH induces closure of the gap junctions, further restricting movement of cGMP into the oocytes. Finally, LH stimulation results in upregulation of AKAP, which sequesters PKA within the mitochondria, preventing its access to the WEE1–MYT1 substrate. LH-mediated alleviation of MPF inactivity results in dephosphorylation and completion of the first meiotic division. (B) Following progression through the second meiotic division, oocytes arrest again in the presence of high CSF activity. CSF is thought to be composed of c-MOS and EMI2 and, together, they ensure suppression of APC/C activity, thus preventing inactivation of the steadily accumulating MPF. Fertilization of the oocyte by sperm results in an influx of calcium ions that, in turn, alleviates this inhibition, resulting in activation of APC/C and subsequent degradation of MPF. (C) Meiotic arrest and resumption are exquisitely timed events, all centering on the activation and inactivation of MPF and CSF activities. This graphical representation highlights key events in the bioactivity of these factors.

this APC/C inhibition, since depletion of EMI2 from egg extracts results in CSF release, while CaMKII, which is upregulated at fertilization, promotes EMI2 degradation, thereby relieving APC/C inhibition and promoting MII exit.[453,454] Importantly, EMI2 is a downstream target of $p90^{RSK}$,[455,456] providing the link between MOS activation and CSF activity.

DISORDERS OF MEIOSIS

Chromosome aneuploidy is a major cause of pregnancy loss and abnormal births in humans. Aneuploidy in fetuses tends to result in spontaneous miscarriage, as the vast majority of these render the fetus nonviable. Even those that survive to birth will have congenital

abnormalities associated with an incorrect chromosome complement. The incidence of genetic aneuploidy in live births is around 0.3%; however, the true incidence of abnormal chromosome number during pregnancy is more likely to be in the region of 35–50%, taking into account those that are stillborn or spontaneously abort.[268,457] Upward of 70% of these errors are thought to arise due to maternal meiosis, with a vast proportion of these as a consequence of defective crossing over during prophase I in humans.[268] In contrast, mouse aneuploidies occur in <2% of meioses, which is surprising given the conservation of meiotic processes in mammalian systems. One reason for this massive discrepancy is likely to be the control of CO frequency and placement, which differs vastly from mouse to human. In mouse oocytes, MLH1 focus numbers are stringently controlled, numbering around 27.5 (±3.7) foci per nucleus.[268,458,459] In humans, in contrast, the number of MLH1 foci in fetal oocytes during pachynema is much more varied, ranging between 10 and 107 per nucleus, and representing a 10-fold difference in frequency from cell to cell, which is evident on the chromosomal level by chromosome arms missing MLH1 foci entirely or being overloaded with MLH1 foci.[268] This variation also differs between individuals, being more severe in some than others. Clearly, this vastly incompetent loading of MLH1 onto female oocytes in the human fetus would have a profound effect on the chromosomes during segregation. Given that these events occur in utero, they are distinct from any etiological causes in postnatal females or in adult women. As such, it is likely that genetic variation and/or changes in the molecular regulation of prophase I events are causes for such alterations in recombination frequency. In addition, it is possible that environmental factors may play a role in inducing this MLH1 variability, leading to the postulation that grandmaternal effects, such as smoking or drug use, may adversely affect meiotic events in female embryos.

Another fundamental cause of the high rate of maternal aneuploidy is the premature breakdown of cohesins associated with meiotic chromosomes in dictyate-arrested oocytes. Given the timing of human female meiosis, in which cohesins are sequestered on the chromosomes during fetal life and must remain there for several years until puberty, it is unsurprising that errors occur. There is evidence from mouse oocytes that once cohesins are recruited to the chromosomes prior to prophase I, they are not regenerated, and thus must maintain cohesion for a considerable period of time, possibly leading to nondisjunction in meiosis I.[460] This cohesin defect is also thought to be a major determinant of female age-related increases in aneuploidy (see below). Less than 10–20% of meiotic errors occur at meiosis II, and these are generally as a result of a precocious loss of centromeric cohesin.[461] Other meiotic genes also might be responsible for high levels of aneuploidy in mammals; a recent study found that mutations in *SYCP3* in females might increase the risks of pregnancy loss due to aneuploidy,[145] and *RNF212* haplotype variation has been implicated as a controlling factor in determining recombination rate in both males and females.[462] Recent evidence has suggested that different patterns of error origin are dependent upon the chromosome in question; for example, errors in separation of chromosomes 16 and 22 more often occur at meiosis II, whereas those in chromosomes 13, 18, and 21 occur at meiosis I.[463]

Maternal Age Effect

As mentioned above, cohesins in mammalian meiosis are thought to play a major role in age-related female aneuploidy, given the comparatively long time between the initial localization of cohesins on chromosomes during prophase I in the female in utero, and the time at which the oocyte emerges from dictyate arrest at the onset of ovulation. For example, *Smc1β* mutant mouse oocytes decrease in fitness with advancing age, with those from one-month-old females showing almost normal meiosis, while those from two-month-old females have vastly increased nondisjunction and meiotic errors.[178] A conditional knockout of *Smc1β*, which prevents SMC1β protein from being synthesized past dictyate arrest around birth, was, however, entirely fertile and showed no age-related loss in fertility, indicating that SMC1β production during prophase I was sufficient to ensure proper chromosome segregation.[464] However, several lines of evidence suggest that this is not the case with other cohesins. REC8 has been implicated as an important regulator of age-related meiotic errors, as it dissociates from chromosome arms and centromeres upon aging, and is not regenerated, leading to destabilized chiasmata and resulting aneuploidy in aged mice. Centromeric cohesin protector SGO2 is also required for proper segregation; however, its localization declines with age in mice.[460,465,466]

CO frequency and distribution are also thought to affect the rate of age-related aneuploidy. In divergent mouse strain crosses (divergence about 1%), aneuploidies in young mice increased relative to controls by around 10-fold, indicating that the sequence divergence was having a profound effect on recombination. In aged mice from the divergent crosses, aneuploidy rose by a further twofold, indicating that the problem was exacerbated in older mice.[467] Studies from human trisomies present a blurred picture as to the extent of influence that CO location has on aneuploidy in humans. One study showed that trisomies from young mothers contained COs more distal or proximal on chromosome 21 than those arising from older mothers; however, another

study claimed that a single pericentromeric exchange correlated with increasing maternal age.[468,469,470] More recent high-resolution mapping of COs in human pedigrees reveals a significant decrease in recombination rate in older women, which is localized around the midsection of the chromosome arms and subtelomeric region.[471] It may be that COs in a certain position render the chromosome susceptible to age-dependent deterioration.

The SAC in human oocytes also may be a cause of age-related increases in aneuploidy. Decreased expression of critical SAC components, such as *Mad2l1* and *Bub1*, has been observed in aged oocytes in humans and mice.[472,473,474,475] However, more recent data from mice reveal no apparent role for SAC in preventing aneuploidy in aged mice, given that MI lasts the same length of time in old and young mice, and they both have an equally robust checkpoint response.[465,476]

Human Diseases Caused by Meiotic Error

Many genetic diseases are caused by meiotic errors, the majority of these resulting from incorrect segregation of chromosomes in the oocytes and sperm. This mis-segregation can result in the offspring carrying too many (trisomy) or too few (monosomy) chromosomes. Typically, full chromosome autosomal monosomies are not found in the human population, but partial chromosome or mosaic monosomies do occur. The most prevalent monosomy in humans involves the deletion of one of the X chromosomes, known as Turner syndrome, which is viable in humans only because of the phenomenon of X inactivation, meaning that many of the cells will still possess a full complement of genes expressed. Diseases caused by chromosomal trisomy are more common in the human population, with the most common of these being trisomy 21, or Down syndrome. Down syndrome sufferers display developmental defects, along with severe mental retardation and a decreased life span. Down syndrome is the most common trisomy disorder seen in humans due to the fact that chromosome 21 is a small autosome, and therefore the trisomic cells can endure this extra chromosomal complement more so than a larger chromosome. Other trisomic diseases have been reported, including Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13), which have varying degrees of disease severity and life expectancy for the sufferer.[477,478] Trisomic disorders involving the sex chromosomes are more common than those involving autosomes, and include XXX, XYY, and XXY (Klinefelter syndrome). In the case of the latter, more than half of documented cases arise from paternal nondisjunction, rather than maternal, which is more common in humans.[479] All patient cohorts of sex chromosome trisomies exhibit some learning and behavioral difficulties as well as fertility problems, and XYY patients often have anger control issues.[480,481]

Environmental Effects on Aneuploidy

There is growing evidence supporting the fact that environmental factors have a large effect on the quality of gametes produced in mammalian species, particularly in oocytes. Diet has long been known to affect oocyte growth and maturation; however, a plethora of recent evidence has indicated the extent to which this is true. High lipid concentrations and maternal obesity have been demonstrated to have a negative correlation with oocyte health, inhibiting germinal vesicle breakdown and preventing the oocyte from proceeding beyond the prophase I stage[482,483]; whereas, conversely, animals maintained on a calorie-restricted diet do not display the usual aneuploidies associated with advancing maternal age.[484]

Smoking is also known to decrease fertility in both men and women, and for women the effect of smoking harms not only their own fertility but also that of their unborn female children.[485,486] Other toxic environmental factors include exposure to the estrogenic chemical bisphenol A (BPA), a component of many plastics and everyday items to which humans are exposed. Experimental evidence from mouse models and rhesus macaques has shown that BPA disrupts meiotic prophase sufficiently to cause synaptic defects, recombinational changes, and nondisjunction in the resulting chromosomes.[487,488] More recently, it has also been shown to have an effect on mouse germline stem cell differentiation, increasing levels of ovarian markers while decreasing levels of testicular markers.[489] So disastrous are these effects that BPA has subsequently been removed from much of the plastic sold in the United States. Temperature changes have also been shown to have an effect on oocyte growth in vitro, whereby the meiotic spindle of human oocytes was disrupted by transient cooling during culture.[490]

CONCLUSION

Our knowledge of the events of mammalian meiosis has evolved rapidly over the past 30 years, driven by impressive advances in our understanding of comparative cytogenetics and genomics, and our ability to extrapolate from similar processes that regulate events such as somatic cell mitosis and DNA repair through gene network analysis. The conservation of events between these processes and across species is remarkable and yet, at the same time, there are features that are unique to mammals, even differing between males and females, which continue to amaze and surprise us.

These differences and similarities across species and sexes encourage and urge us to use comparative approaches at all times to elucidate the mechanisms of meiotic regulation. The next frontier in our understanding will come from continued understanding of the molecular pathways involved in recombination and checkpoint control, and how the epigenetic and RNA regulatory events integrate with those pathways. For example, our understanding of how DSB events are regulated at the chromatin level through *Prdm9* action has radically changed the way we think about DSB placement and CO assurance. More surprising has been the evidence from canids showing that, in the absence of a functional *Prdm9* allele, DSBs are directed to GC-rich regions and are evolutionarily more stable than recombination hotspots in other organisms.[296,297] This has led to speculation that recombination in the dog may be controlled by an ancestral mechanism that is found in other mammalian species but is outcompeted for in terms of DSB repair resources by the PRDM9-driven mechanism. What has also become clear in recent years is that CO placement is controlled by three distinct mechanisms: not only by interference, as had been previously thought, but by chromatin structure and CO homeostasis as well. The future in this area of research is particularly exciting. With exciting technologies to study recombination mechanisms, using methods such as ChIP-seq and SPO11-associated oligo mapping, already emerging, it seems the high-throughput approach to studying recombination is the direction that future studies will take. In addition, with the prospect of fast and inexpensive whole genome sequencing on the horizon, recombination events could be mapped over entire genomes in individual organisms or cells in the not-too-distant future.

A unique feature of mammalian, and specifically male, meiosis is the mechanism of meiotic silencing, particularly of the sex chromosomes. What has emerged in recent years is a picture whereby MSCI is inextricably linked to recombination and synapsis through the availability of specific mediators of each process, and these mediators (i.e., BRCA1 and ATR, but there may be others) act through heterochromatin formation and potentially utilize small RNA-driven mechanisms. This paradigm establishes an exciting interrelationship between gene-coding and noncoding mechanisms acting at the level of the DNA to regulate meiotic processes, and the elucidation of these RNA-based interactions over the coming decades is likely to change the meiotic landscape considerably.

Increasingly, studies specifically into meiotic processes in mice have helped advance similar studies in humans, and have led to major changes in the way we view human health. Advances in aneuploidy research and the massive effects that environmental factors can have on both male and, particularly, female meiotic health have led to changes in the way people choose to live their lives. The "grandmaternal effect" of exposing our unborn daughters to factors that may influence their reproductive health in years to come has changed international government policies on using these toxins in our plasticware. Research into female meiosis and the possible existence of oogonial stem cells isolated from both aged mice and humans has challenged the central dogma in reproductive biology stating that only a finite pool of oocytes exists at birth, and that this pool is never added to in later life. This area of reproductive biology remains controversial, but the broader questions of how germline stem cells become competent to undergo meiosis and, more importantly, how they do so with the exceptionally high fidelity needed to ensure the success of sexual reproduction represent an exciting area of continued and future research. Elucidating the molecular basis for meiotic entry and accurate progression has huge implications for our understanding of why human females exhibit such high rates of meiotic errors. Indeed this disparity between species in meiotic success rates remains one of the most poorly understood disease-related phenomena in human fertility despite the strict conservation of meiotic processes across eukaryotes.

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CHAPTER

2

The Mammalian Oocyte

David F. Albertini

Department of Molecular and Integrative Physiology, Institute of Reproductive Health and Regenerative Medicine, Kansas University Medical Center, Kansas City, KS, USA

INTRODUCTION

The oocyte is distinct from all other metazoan cells by its ability to become a totipotent zygote. This fundamental property of female germ cells is not only retained in mammals but has required adjustments in design and function to meet the demands of the wide variety of reproductive strategies exhibited in mammals. While diversity between mammalian species exists with respect to the time required for oocyte development, there is remarkable conservation in basic processes governing the differentiation of a cell capable of effecting fertilization and embryogenesis. As stated above, among the most unique properties of the mammalian oocytes is the ability to generate totipotency. While subtle differences exist between mammalian species, the ability of the oocyte to reprogram the reconstituted diploid genome of the zygote, or that of a differentiated somatic cell nucleus, into a complete organism has been the culmination of researches encompassing more than two centuries.

Many historical accounts of the mammalian oocyte have been published, and for reaching into the deep past in this area, the reader is encouraged to examine "The Ovary of Eve".[1] That the ovum of vertebrates constitutes the generative cell was first suggested by William Harvey by the oft-cited statement, "*ex ovo de vivum*". It has taken many years to fully appreciate the ovum's totipotent character, requiring in retrospect the realization that the oocyte is contained within the ovarian follicle and undergoes a process of "maturation" as it is released from the ovary at ovulation, and finally accepting that fusion of egg with sperm launched a transformation of the ovum into the zygote, from which the embryo would develop.[2] These initial milestones are recorded in great detail in E. B. Wilson's treatise "The Cell in Development and Heredity," where the first glimpses of the process of oogenesis were described for the many studies on invertebrate animal models readily accessible in quantity and quality to analysis at the microscopic level.[3,4] Most important, these classical comparative studies on the oocyte formed the foundation upon which the basic principle that oogenesis presupposes embryogenesis was firmly established.

Appreciation that the oocytes of vertebrates more broadly applied to the biology of early development awaited the observational studies of the mid-twentieth century, where both the commonalities and distinctions of the oocytes of mammals were contributed by the likes of Austin, Beams, and Short. Among the principles reinforced from this era of research were: (1) the realization that the initial energy reserves for the embryo were deposited in the oocyte; (2) the appearance of extracellular coats for protection of the embryo and establishing the block to polyspermy; (3) the recognition that meiosis proceeds from diplotene to metaphase two in a highly developmental sequence under hormonal control; and (4) the interrelationship of oogenesis and folliculogenesis. This latter point was debated with vigor as the work of Sir Solly Zuckerman proposed that female mammals were born with a finite supply of oocytes that gradually diminished with advancing maternal age. The next significant phase of discovery took place in the latter half of the twentieth century, as has been summarized by Biggers.[4,5] Insights into the ultrastructure of the mammalian oocyte and the regulation of the meiotic cell cycle were obtained during this period of research.

Electron microscopic investigations defined in great detail the variety of organelles that constitute the "mother lode" of information that will carry the embryo to and through early development.[6,7] As shown in Figure 2.1, these descriptive studies revealed that the continuity of organelles through the female germ line to the embryo embodied mitochondria, the Golgi complex, endoplasmic reticulum, ribosomes, cortical granules,[8] and more specialized structures such as annulate lamellae (not

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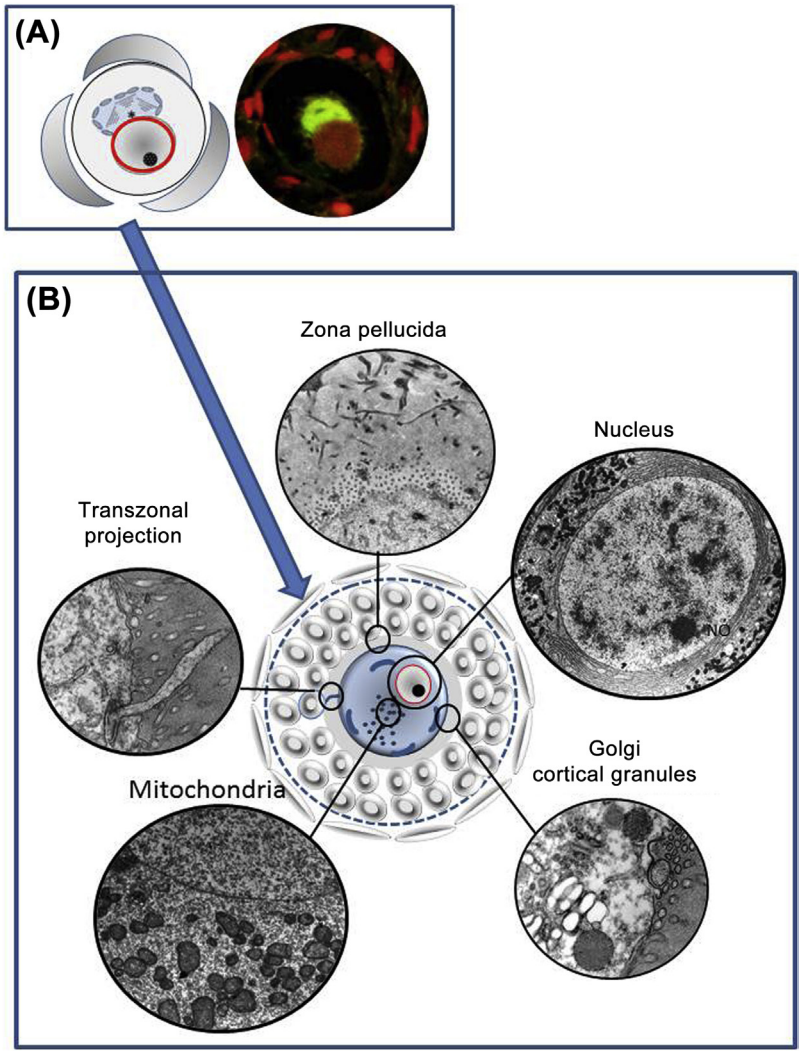

FIGURE 2.1 Structure of the mammalian oocyte. This figure is reproduced in color in the color plate section. (A) depicts the general organization of organelles in a primordial follicle; schematic (left) denotes centrally positioned nucleus and aggregation of organelles known as Balbiani's body (asterisk). Right is an immunofluorescence micrograph illustrating the concentration of the germ cell–specific marker VASA within Balbiani's body (green) located next to the oocyte nucleus (red). *(Image courtesy of Professor Alfredo Vitullo)*. Upon activation, oocytes enter the growth phase of oogenesis (B). (B) summarizes the major ultrastructural features of a growing oocyte within a preantral follicle noting the assembly of the extracellular coat or zona pellucida, the enlarged nucleus or germinal vesicle with prominent nucleoli (NO), subcortical Golgi complexes with associated cortical granules, abundant perinuclear mitochondria, and the elaboration of microvilli on the oocyte plasma membrane interacting with somatic cell projections known as transzonal processes.

shown), and cytoplasmic inclusions of various kinds that often were unique to certain mammalian species.[9] Moreover, ultrastructural studies reinforced and extended mechanistic thinking as it pertained to interactions of somatic cells with the oocyte and alterations in the structure and function of the oocyte plasma membrane known as the oolemma.

Concurrent with ultrastructural investigations, technologies were being honed that would permit the isolation and culture of oocytes, ova, and embryos, opening up an era of hypothesis-driven research making possible studies coupling structure with function. The foundations for this field were made by Gregory Pincus and colleagues at the Worcester Foundation for Experimental Biology during the 1930 and 1940s.[10] Although rarely acknowledged, Pincus' pioneering work established in vitro oocyte maturation (IVM) technology.[11] Some 20 years later, Bob Edwards established the utility of IVM for many mammalian species that conceptually laid the framework for much of what we know about the meiotic cell cycle today and led to the practice of human in vitro fertilization as we know it today.[12,13] Finally, experimental strategies for the ex vivo manipulation and analysis of virtually all stages of oogenesis in the mouse were established.[5,14] It was at this point that research flourished using the mouse model, as evidenced by the appearance of a number of papers that established the foundation for biochemical and molecular investigations of mammalian oogenesis.[15–18]

Important discoveries soon followed. For example, direct evidence documenting the dependence of the mouse oocyte on cumulus cell metabolism[19] was obtained. Work from Bob Moor's group validated the use of in vitro techniques in sheep oocytes, allowing for a detailed analysis of protein synthesis changes and meiotic cell cycle progression kinetics during IVM.[20–22] Importantly, these studies established the impact of culture on oocyte quality and developmental competence with respect to hormonal factors that remain relevant to

TABLE 2.1 Competencies Acquired by Mammalian Oocytes during Oogenesis

Competency	Stage Acquired	Function
Meiotic	Late G	Resumption and completion of meiosis
Imprinting	Late G	Methylation of imprinted genes
Cortical granule fusion	M	Block to polyspermy
Calcium oscillations	M	Egg activation
Male pronucleus	M	Protamine S–S reduction; histone exchange
Cleavage	PM/M	Ability to sustain blastomere divisions
Compaction	PM/M	Ability to undergo cell polarization and lineage allocation
Embryonic stem cell derivation	G/PM/M	Ability to propagate stem cells from inner cell mass
Term birth	G/PM/M	Ability to sustain term gestation

Derived primarily from studies in the mouse based on phases of oogenesis described in Figure 2.2. Abbreviations: G, growth phase; PM, prematuration; M, maturation phase.

human assisted reproductive technologies (ARTs) to this day. Patterns of RNA synthesis were documented,[22] and the first studies describing an inhibitory role for adenosine 3′,5′-cyclic monophosphate (cAMP) in maintaining meiotic arrest appeared[23] (see Chapter 1). This discovery, the first to probe the mechanisms of cell cycle control during mammalian oogenesis, has become a centerpiece for much of the work exploring the relationship between cumulus cells and the oocyte over the past 40 years. Moreover, experiments of this kind paved the way for asking important clinical questions as to why some oocytes are able to develop after fertilization and why some fail. Whether mammalian oocytes are able to enact the events leading to successful embryonic development has been operationally defined as competence. Throughout this chapter, reference will be made to the oocyte competencies acquired at specific stages of oogenesis, as shown in Table 2.1.

The background literature up to this point sets the stage for much of the content of this chapter. While any attempt to cover the topic mammalian oocytes would fall far short of recognizing the many important contributions made prior to the "molecular genetic" era, there are several central tenets in the biology of the oocyte that have withstood the test of time and form recurrent themes of great importance to reproductive physiology.

The first theme embodies the notion that embryogenesis begins with oogenesis.[24,25] Maternal effect genes, products of oogenesis required for embryonic development, have been identified as have a variety of non-coding RNAs that are likely to play a role following fertilization.[26,27] Many of the proteins, and their posttranslational modifications, exerting control of the cell cycle from mitosis to meiosis and back to embryonic mitoses have been characterized (see Chapter 1). Together, the cytoplasm of the mammalian oocyte has been more critically defined in molecular terms, and to no surprise, the changing requirements at the protein level have taken on an aura of complexity that typifies the changing landscape within the oocyte as it proceeds through oogenesis. Figure 2.2 provides a framework for this chapter.

As Figure 2.2 illustrates, the process of oogenesis begins in the fetal ovary with the assembly of primordial follicles after the transition of primordial germ cells into meiotic prophase (see Chapter 1). With the formation of primordial follicles, oocytes have entered the diplotene stage of prophase one and will remain arrested in the cell cycle until luteinizing hormone (LH) induces ovulation and the resumption of meiosis. For the purposes of this chapter, oogenesis is divided into the growth phase, the pre-maturation phase, and finally, the maturation stage as shown in Figure 2.2. It is important to first appreciate that as the oocyte progresses through oogenesis, it maintains a temporal relationship within the ovarian follicle ensuring that the two processes, oogenesis and folliculogenesis, are integrated (see Chapter 21). When the primordial follicle is activated, oocyte growth proceeds in tandem with follicle growth in a gonadotropin-independent fashion. Once the follicle becomes sensitive to the action of gonadotropins, oocytes make the transition from the growth to pre-maturation phases, signaling the onset of the gonadotropin-dependent phase of folliculogenesis. As will be discussed below, the major factors that regulate development during the pre-antral growth phase involve paracrine and gap–junction mediated signaling mechanisms. In antral follicles, follicle-stimulating hormone (FSH) promotes growth and differentiation of somatic cells, ensuring that they eventually acquire sensitivity to LH, the primary trigger for ovulation and oocyte maturation. Each of these phases has been identified as error-prone in terms of nuclear (genetic, epigenetic) or cytoplasmic quality and have been implicated as determinants of embryonic developmental competence.[24,25,28–30]

Thus, the second major theme implies that oogenesis and folliculogenesis are inseparable from one another and any deviation from their coordination and integration spells failure to the reproductive fitness of a given species (see Chapters 21 and 22). From the vantage point of the oocyte, the growth phase of oogenesis relies heavily on direct forms of paracrine signaling between the oocyte and

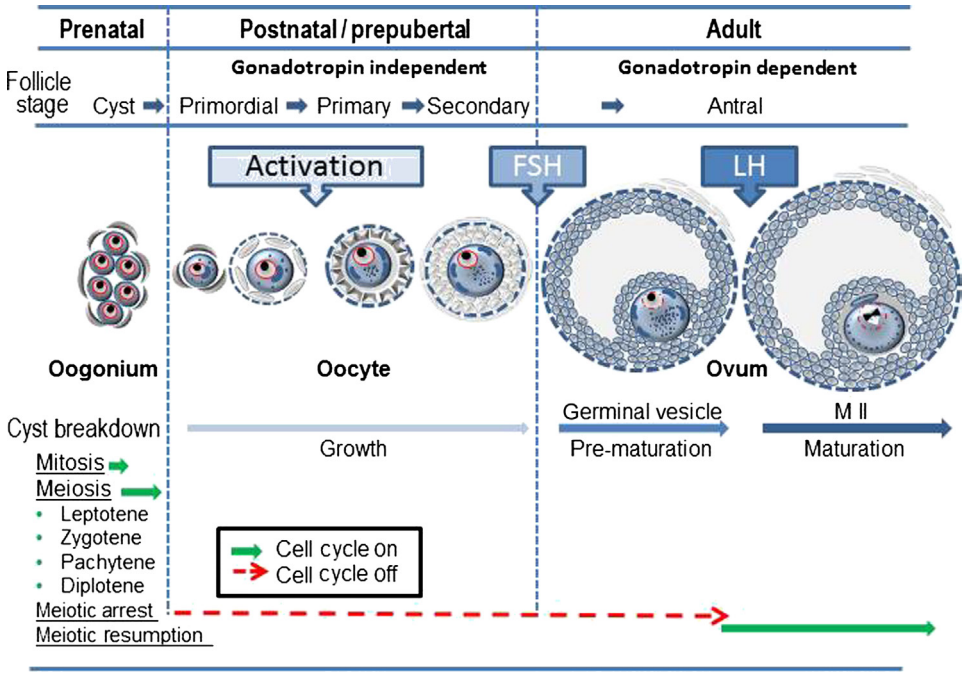

FIGURE 2.2 **Stages of oogenesis defined on the basis of the oocyte cell cycle state as it occurs in the mouse.** This figure is reproduced in color in the color plate section. Mitotic proliferation of oogonia occurs in the prenatal gonad and is accompanied by entry into meiotic prophase as follicle formation and cyst breakdown take place. The cell cycle is then turned off for the duration of the growth phase of oogenesis that occurs during the preantral stages of follicle development. While FSH is required to advance follicle development, the oocytes remain in meiotic arrest with a germinal vesicle in the prematuration stage. In response to the LH surge, meiotic arrest is released and the oocyte proceeds through maturation being ovulated at the metaphase two stage of meiosis, which is maintained until the fertilization signal elicits completion of meiosis two. Note that in general the growth phase of oogenesis occurs independent of gonadotropin stimulation, whereas the prematuration and maturation phases require gonadotropin stimulation.

surrounding somatic cells without direct involvement of gonadotropins (Figure 2.2). For basic and clinical scientists alike, the notion that the oocyte maintains communication with the soma throughout the various stages of follicle development has great significance.[31] The communication pathways at the interface of cumulus cells and the oocyte are now understood to involve many different forms of intercellular signaling that impact the development of both cell types.[32] Moreover, such communication pathways integrate germline and somatic cells to the overall benefit of embryo quality and the establishment of pregnancy.[33] There is good evidence to support the idea that the impact on the embryo is realized by epigenetic regulation. Importantly, epigenetics plays a role in separating genetic regulation of oocyte differentiation from that of the process of meiosis.[34] And during the growth phase of oogenesis, the expression of totipotency factors such as *Oct4* is modulated by gonadotropins through specific histone alterations.[35]

The third and final major theme for this chapter regards the problem of ovarian aging and the associated loss of oocyte quality. Humans are unique among mammals in the propensity with which their oocytes obtain an aneuploid condition, especially with advancing maternal age. Why our species is uniquely susceptible to genetic instability is not understood, but current opinion advocates faulty control of the meiotic and mitotic cell cycle.[36] Moreover, recent evidence suggests that other forms of genetic instability involving translocations, inversions, and DNA damage repair mechanisms are at play and that the oocyte itself may have a limited corrective potential for rectifying defects inherited paternally or maternally. Potential links between lifestyle alterations, environmental contaminants and reproductive failure have been proposed to find causality in these forms of genetic instability, clinically manifest in the high incidence of mosaicism observed in human embryos.[37]

Thus, in providing scope for this chapter, the link between oogenesis and embryogenesis, and the integration of oogenesis and folliculogenesis bear directly on the problem of ovarian aging and the clinical ramifications now being confronted in the context of human health and disease. This raises the interesting question as to the meaning of commonalities and differences in mammalian oocytes when compared between species. As shown in Figures 2.1 and 2.3, many structural properties exhibited by mammalian ova are conserved, such as the large size of the nucleus, prominent nucleoli, the presence of cortical granules, and the zona pellucida. Many of these properties are shared with the oocytes of lower vertebrates and invertebrates.[38] Where possibly of importance, attention

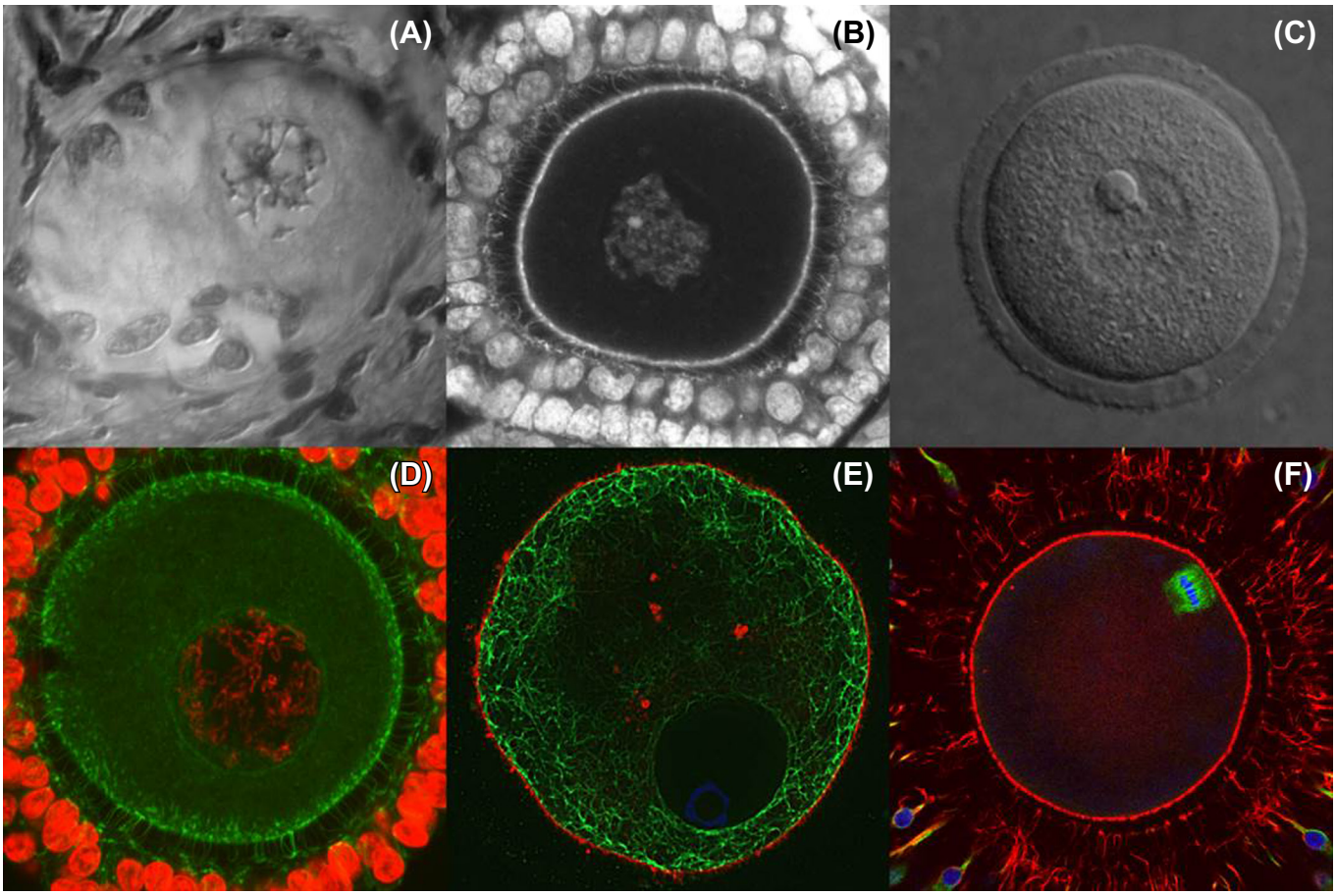

FIGURE 2.3 Prominent morphological features of the mammalian ovum at various stages of oogenesis in various species. This figure is reproduced in color in the color plate section. (A) depicts a resting primordial oocyte within the bovine ovary in which the germinal vesicle chromatin assumes a distinctly fibrillar pattern of organization. (B) represents a primary growing oocyte from the mouse containing a centrally positioned germinal vesicle and illustrating the prominent actin-rich cortex and numerous transzonal projections emanating from surrounding granulosa cells. (C) demonstrates the appearance of a full-grown immature mouse oocyte following isolation from the ovary; note the prominent eccentrically positioned nucleolus within the GV and the cell free zona pellucida. (D) depicts a full-grown immature bovine oocyte isolated from a Graafian follicle; note the clear organization of bivalents in the GV and the presence of numerous foci at the oocyte cell surface, which represent terminal connections with corona TZPs (preparation labels f-actin using rhodamine phalloidin). (E) illustrates an immature GV-stage human oocyte that has been labeled for chromatin and microtubules (fibrillar structures); note that the chromatin is aggregated around the nucleolus of the GV and that a dense network of cytoplasmic microtubules is present in the outer regions of ooplasm. (F) illustrates a mature metaphase two arrested oocyte from a horse following controlled ovarian hyperstimulation; this preparation has been dual-labeled for tubulin (green) that highlights the cortical anchored meiotic spindle and f-actin (red) that labels the prominent corona cell projections or TZPs that appear detached from the oolemma.

will be drawn to variations in mammalian oocytes that aid in understanding their physiology.[39]

CELL BIOLOGY OF THE OOCYTE

By far, one of the most striking features of the mammalian oocyte is its size. The transition from a quiescent non-growing state within the primordial follicle to that of active growth is one of the most dramatic examples of cellular hypertrophy known. Expansion in size occurs as a consequence to robust gene activation, selective protein synthesis, and hyperplasia of organelles. Of the new proteins and RNAs synthesized during the growth phase are those required for housekeeping and sustaining hypertrophy, those involved with regulation of the meiotic cell cycle, and those whose function will not be realized until later stages of development, so-called "maternal effect genes". Thus, the oocyte, arrested in diplotene of prophase in meiosis 1 (Chapter 1), exhibits a burst of transcriptional activity upon primordial follicle activation that persists until the oocyte has completed growth, as was first demonstrated in the mouse.[40] The unique challenge posed in the oocyte is how to selectively translate or store maternal RNAs of various classes, ensuring the readout of transcripts temporally restricted to the growth phase, cell cycle transitions, or after fertilization. Compounding this phase of development are emerging