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Preface

This volume of *Current Topics in Developmental Biology* is remarkable for the breadth and depth of research described in the expanding field of molecular development. From germ cell structure, to a new approach to gene-environment interaction, to epigenetics, to mitochondrial function and dynamics, this volume is exceptional for its “micro” approach to “macro” issues. Developmental biologists are fortunate to enjoy a rich and important scientific history now a few centuries old, culminating here in outstanding expositions on our science. Our first chapter considers germ cell origins; the second and third grapple with the molecular determinants of complex adult behavior. The last two contributions consider mitochondria’s invaluable role in development.

The Balbiani Body and Germ Cell Determinants: 150 Years Later by Malgorzata Kloc and Laurence Etkin of the MD Anderson Cancer Center, and Szczepan Bilinskil of Jagiellonian University, describes the cytoplasmic structure containing germinal plasm. Drawing on data from a host of new-found molecular markers in germ plasm, germinal granules, nuage, and the Balbiani body, the authors describe germ cell structures in over a dozen species, suggest unified nomenclature, and posit common characteristics and functions for the structures in these many organisms.

In Fetal-Maternal Interactions: Prenatal Psychobiological Precursors to Adaptive Infant Development, Matthew Novak of the National Institute of Child Health and Human Development applies Sackett’s work on postnatal adaptation to the prenatal environment, pointing out that many adaptive behaviors deemed “postnatal” actually originate before birth as responses to fetal “experiences” (conditions that others might define as fetal “stress”). Extending the effects of pre- and postnatal environments relative to genetics, Novak stresses how critical it is for developmental biologists to consider how intervention in the earliest stages of growth (by manipulating stem cells, transfecting genes, or employing assisted reproductive technologies) may affect experimental organisms.

Paradoxical Role of Methyl-CpG-Binding Protein 2 in Rett Syndrome by Janine LaSalle of the University of California School of Medicine summarizes findings (and new questions) arising in the five years since this devastating developmental disability was shown to be caused by a mutated *MECP2* gene, which is now known to interfere with proper CpG methylation, chromatin organization, and ultimately with central nervous system development. Elucidation of this one relatively rare disorder provides a model for showing how epigenetic events are crucial to normal mammalian brain development.
Genetic Approaches to Analyzing Mitochondrial Outer Membrane Permeability by Brett Graham and William Craigen of the Baylor College of Medicine examines voltage-dependent anion channels (VDACs), the proteins that form pores in the outer membranes of eukaryotic mitochondria, the exact function of which is unclear. For a more complete picture, the authors provide a comprehensive review of the literature regarding VDAC-deficient yeast, fruit flies, and mice, varied species that display similar phenotypes when missing VDAC isoforms. This is, in a sense, an experimentalist’s cautionary tale, as much of what is known about VDACs in vitro does not resemble what is now being learned about VDACs in vivo. The authors are optimistic that Drosophila will prove to be a suitable model organism for VDACs in vivo.

Mitochondrial Dynamics in Mammals by Hsiuchen Chen and David Chan of the California Institute of Technology examines mitochondrial fission and fusion. When the rates of these events are changed, subsequent changes in mitochondrial populations can have profound changes on the organism, with implications for development, disease, and – particularly intriguing – cellular suicide.

This volume has benefited from the ongoing cooperation of a team of participants who are jointly responsible for the content and quality of its material. The authors deserve the full credit for their success in covering their subjects in depth yet with clarity, and for challenging the reader to think about these topics in new ways. The members of the Editorial Board are thanked for their suggestions of topics and authors. I also thank Laura Hewitson and Leah Kauffman for their fabulous scientific insights and Rhonda Genes for her exemplary administrative support. Finally, we are grateful to everyone at the Pittsburgh Development Center of Magee-Womens Research Institute here at the University of Pittsburgh School of Medicine for providing intellectual and infrastructural support for Current Topics in Developmental Biology.

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The Balbiani Body and Germ Cell Determinants: 150 Years Later

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I. Introduction

In most animal species, the germ cells segregate from the soma early in embryonic development. As a rule, germ cell fate depends on the acquisition of a germ cell determinant commonly called the germ plasm/germinal plasm/polar plasm/pole plasm/oosome/nuage. The term pole plasm was introduced
in 1908, by Kahle, for the description of uniquely stained cytoplasm in the pole (germ) cells of dipteran insects, and subsequently, in 1931, the term germ plasm was used by Bounoure to describe similar cytoplasmic structures in frog eggs and in primordial germ cells of frog embryos. The germ plasm is an indispensable element of germ cell identity. It was shown that the experimental inactivation of the germ plasm in developing embryos either by ablation or ultraviolet irradiation resulted in either a decrease in the number of functional gametes or complete sterility (Bounoure, 1937; Buehr and Blackler, 1970; Geigy, 1931; Hegner, 1914; Illmensee and Mahowald, 1974; Smith, 1966). In contrast, the transplantation of the germ plasm or its components from its normal position to another part of the embryo resulted in the ectopic production of germ cells (reviewed in Eddy, 1975; Ephrussi and Lehmann, 1992; Illmensee and Mahowald, 1976; Mahowald, 1962; Underwood et al., 1980; Wakahara, 1978). The germ plasm contains a set of unique, morphologically and ultrastructurally identifiable structures that over the years have been given various names such as P granules in Caenorhabditis elegans, polar granules in Drosophila and other insects, and germinal granules in Xenopus (al-Mukhtar and Webb, 1971; Counce, 1963; reviewed in Eddy, 1975; Strome and Wood, 1982).

It is well established that the germinal plasm in the oocytes of Xenopus is located in the specialized cytoplasmic structure called the Balbiani body (De Smedt et al., 2000; reviewed in Guraya, 1979; Heasman et al., 1984; Kloc et al., 2001a; Tourte et al., 1981). The structure was first reported in 1845, by von Wittich, in the oocytes of spiders, and was given name of “vitelline (yolk) nucleus” (“Dotterkern”) by Carus in 1850. Comprehensively studied by French embryologist E. G. Balbiani between 1864 and 1893 in oocytes of myriapods and spiders, it was given, in 1887, the name of “Balbiani vitelline (yolk) body” or “Balbiani body” by Balbiani’s student Henneguy (Fig. 1). Over many decades, the terminology used to describe the Balbiani body (Bb) in various animal species became as chaotic and confusing as the nomenclature used to describe the germ plasm. In most cases, the name Bb has been used interchangeably with terms such as mitochondrial mass, mitochondrial aggregate, mitochondrial cloud, yolk nucleus, yolk nucleus complex, or vitelline (yolk) body (reviewed in Guraya, 1979). In addition, in some instances, the Bb has been confused with and described either as the germ plasm itself or the nuage (De Smedt et al., 2000; reviewed in Guraya, 1979; Kloc et al., 2001a), and it has been equated with the sponge-like bodies in nurse cells of Drosophila (Wilsch-Brauninger et al., 1997). The fact that the molecular composition of all of these structures and their function are often hypothetical or only sketchily understood adds an extra level of perplexity to these descriptions. The chaos in the nomenclature makes it very difficult or nearly impossible for readers of the earlier and later publications to follow and compare the relationships among all these structures in different species. Since Guraya in 1979 wrote the most comprehensive review
Figure 1 Balbiani body in the oocytes of arachnids and myriapods. (A) Domestic spider Tegenaria domestica. Late previtellogenic oocyte with prominent Bb (arrow). Semithin section, methylene blue. (B and C) Pseudoscorpion Chelifer sp. (Courtesy of Dr. J. Kubrakiewicz, University of Wroclaw.) (B) Two early previtellogenic oocytes with Bbs (arrows). Cisternae of endoplasmic reticulum (arrowhead). Semithin section, methylene blue. (C) EM micrograph of an early previtellogenic oocyte. Nuage (arrow) is visible between mitochondria (m). Endoplasmic reticulum (arrowhead). (D) Primitive myriapod, Hanseniella sp. EM micrograph of a fragment of Bb in early previtellogenic oocyte. Nuage (arrow), mitochondria (m). Nucleus (n). Scale bars: (A) 40 \mu m; (B) 20 \mu m; (C) 1.6 \mu m; (D) 1 \mu m.

To date that indexed the ultrastructural and cytochemical data on Bb and related structures in the oogenesis of invertebrates and vertebrates, it was not our intention to replicate or just simply to update this review. However, various molecules (RNAs and proteins) have been discovered within the germ plasm, germinal granules, nuage, and Bb (reviewed in Houston and King, 2000a; Kloc et al., 2001a; Mahowald, 2001; Seydoux and Schedl, 2001). These discoveries handed us a set of molecular markers that could be used to compare the relationships among all these structures. This prompted us to compile the most recent ultrastructural, molecular, and functional data on germ cell-specific structures in different species and to try logically to
organize and unify the nomenclature and, most important, to assess a possible homology in composition and function among these structures in different animal taxa and species.

Because the most comprehensive ultrastructural and molecular studies on the origin, composition, and function of Bb and its relationship to the germ plasm have been done for the oocytes and embryos of *Xenopus*, we start our description with *Xenopus* and use *Xenopus* nomenclature as a paradigm.

Although we realize that the old nomenclature, albeit confusing and chaotic, will be impossible to replace, at least in the case of such organisms as *C. elegans*, *Drosophila*, or *Xenopus* for which publications on the subject have accumulated for decades, we hope that the more consistent terminology can be used in the reports of future discoveries. For historical reasons, we should keep unchanged the terms P granules in *C. elegans* and other worms, polar granules in *Drosophila* and other insects, and mitochondrial cloud in *Xenopus*. However, for future descriptions, we propose to employ the following generic terminology: nuage—for the description of electron-dense material located in the vicinity of the nuclear membrane and/or traversing the nuclear pores; mitochondrial cement—for only the nuage closely attached to mitochondria; germinal granules—a generic term for all electron-dense material present in the cytoplasm away from the nucleus; germ plasm islands, which may contain germinal granules—for the distinct regions of cytoplasm visible in the cortex of stage VI oocyte and in the cleaving embryo; and Balbiani body or mitochondrial cloud—for the structures that surround and translocate the germinal granules and/or mitochondria toward the oocyte cortex and that should be clearly distinguished as transporting vehicles and should not be called a nuage, germ plasm, or germinal granule.

II. *Xenopus*

A. Origin, Behavior, and Ultrastructure of the Balbiani Body

Over the years, the Bb in *Xenopus* has been referred to, in most studies, as the mitochondrial cloud (MC) and much less frequently as the mitochondrial mass or aggregate (al-Mukhtar and Webb, 1971; Billett and Adam, 1976; Coggins, 1973; Heasman et al., 1984; reviewed in Kloc et al., 2001a; Tourte et al., 1981; Wylie et al., 1985). In stage I oocytes the MC is a spherical (approximately 40 μm in diameter) structure in contact with the oocyte nucleus (Fig. 2). The MC always faces a future vegetal pole of the oocyte and thus is a distinct marker of the animal/vegetal polarity of the oocyte. In stage I oocytes, the MC is composed of approximately 500,000 mitochondria (Marinos and Billett, 1981), hence the term a “mitochondrial cloud.” The mitochondria within the MC differ in many aspects, such as morphology,
Figure 2  Diagram of mitochondrial cloud formation and dispersion in *Xenopus* oogenesis and embryogenesis. (1) In prestage I and early stage I oocytes, nuage material (marked in pink) leaves the nucleus and comes into close contact with mitochondria (green), thus becoming the mitochondrial cement within the primary mitochondrial cloud (PMC). (2) In stage I oocytes, a round mitochondrial cloud (MC) is located in the vicinity of the oocyte nucleus. Germinal granules are concentrated in the METRO region of the MC. (3) Between stages II and IV of oogenesis, the MC fragments into islands that move toward the vegetal cortex. (4) Between stages IV and VI of oogenesis, the germ plasm islands (remnants of the MC) anchor in the vegetal cortex of the oocyte. (5) After fertilization and during early cleavages, germ plasm islands coalesce into larger aggregates at the vegetal apexes of vegetal blastomeres. (6) In late blastula and in gastrula, the group of primordial germ cells (PGCs) is visible within the embryonic endoderm. PGCs contain nuage in the vicinity of their nuclei and loosely arranged mitochondria.
enzymes activities, and replication properties from the mitochondria in other regions of the ooplasm. This suggests that the MC in Xenopus contains a germ line-specific subset of mitochondria that are ultimately delivered to the germ plasm in the embryo and primordial germ cells (PGCs) (D’Herde et al., 1995; Marinos and Billett, 1981; Mignotte et al., 1987; Tourte et al., 1981, 1984). Other components of the MC are the rough endoplasmic reticulum, numerous Golgi complexes (distributed more or less uniformly throughout), and electron-dense granulofibrillar roundish structures called the germinal granules. The germinal granules are concentrated at the vegetal apex of the MC in the region called the message transport organizer (METRO) (Fig. 3) (Kloc and Etkin, 1995). There are approximately 700 germinal granules, ranging between 50 and 2000 nm in diameter, in the METRO (reviewed in Kloc et al., 2001a; Kloc et al., 2002). During oocyte growth (between stage II and stage VI), the MC fragments into many small islands that contain all the components of the MC, including the germinal granules and mitochondria. The islands relocate toward the vegetal cortex. It is unknown how the islands of MC move toward the vegetal pole of the oocyte. It is possible that this is a passive movement caused by the streaming of the cytoplasm toward the vegetal cortex, but the definitive answer awaits further study. The islands of MC anchored at the apex of the vegetal cortex of stage VI oocytes are often described as the germ plasm islands, or germ plasm. It seems that the anchoring of the islands of the MC and localized RNA molecules (see below) at the vegetal cortex depends on actin filaments (Kloc and Etkin, 1995). The germ plasm islands in the vegetal cortex of stage VI oocytes contain hundreds of small germinal granules (250–500 nm in diameter), numerous mitochondria and vesicles and cisternae of Golgi, and endoplasmic reticulum (ER) (Czolowska, 1969; reviewed in Kloc et al., 2001a). After fertilization, the germ plasm islands coalesce using the Xklp kinesin-like protein as a motor, into larger units. This is accompanied by the formation of larger germinal granule aggregates (Kloc et al., 2002; Quaas and Wylie, 2002; Robb et al., 1996). Subsequently, during cleavage, the germ plasm with its germinal granules and mitochondria segregates to the vegetal blastomeres by ingressing along the cleavage furrows. The germ plasm islands of an 8-cell embryo contain about 80 large (2000 nm in diameter) germinal granules situated between the mitochondria (Kloc et al., 2002). Ultimately, the germinal granules and germ plasm islands fragment again and segregate into PGCs. When PGCs move toward the gonad rudiment, the germinal granules disappear and become replaced by the nuage that forms in the vicinity of the nuclear envelope (reviewed in Houston and King, 2000a; Kloc et al., 2001a).

It seems, although detailed studies are scarce, that other anurans such as Rana also have a MC in their oocytes and develop germ cells by a
Figure 3  Ultrastructural aspects of the mitochondrial cloud in *Xenopus* oocytes and embryos. (A–D) EM micrographs. (A) Fragment of an early stage I oocyte. Mitochondrial cement (arrows) is visible between mitochondria (m) and in contact with the nuclear envelope. (Compare with Fig. 2, part 1.) n, Nucleus. (B) METRO region of mitochondrial cloud of stage I oocyte, showing the aggregation of germinal granules (arrows) between mitochondria (m). (Compare with Fig. 2, part 2.) (C) Fragment of the vegetal pole of late stage II oocyte. Islands of dispersing mitochondrial cloud contain mitochondria (m) and germinal granules (arrows).
mechanism similar to that of *Xenopus* (reviewed in Guraya, 1979; Mahowald and Hennen, 1971; Williams and Smith, 1971).

The formation of the MC can be traced to the oogonia and prestage I oocytes in the developing ovary of the *Xenopus* froglet. Each primary oogonium (cystoblast) has a lobulated nucleus and an oval or kidney-shaped mitochondrial aggregate that is a precursor of the primary mitochondrial cloud (PMC) (al-Mukhtar and Webb, 1971; Coggins, 1973; Kloc et al., 2004; Tourte et al., 1981). Secondary oogonia (cystocytes) and prestage I oocytes in the early pachytene of the first meiotic prophase have a spherical PMC that is a precursor of the MC of stage I oocytes (al-Mukhtar and Webb, 1971; Coggins, 1973; Kloc et al., 2004; Tourte et al., 1981). The mitochondrial aggregate and PMC contain numerous mitochondria, ER, and Golgi complexes and abundant microtubules—all of them arranged around a centrally located pair of centrioles and a germ line-specific vesicular structure called the fusome (Kloc et al., 2004). In addition, the mitochondrial aggregate and PMC contain irregularly shaped accumulations of electron-dense material called the mitochondrial cement that is located between and in close contact with the mitochondria. In older literature, the mitochondrial cement was often referred to as the nuage (al-Mukhtar and Webb, 1971; Coggins, 1973; Heasman et al., 1984; Kloc et al., 2004; Tourte et al., 1981; Wylie et al., 1985). It is believed that the mitochondrial cement originates in the nucleus. Small patches of nuage material similar in appearance to mitochondrial cement were often reported traversing the nuclear pores and accumulating in the vicinity of the nuclear membrane before coming into contact with mitochondria and becoming mitochondrial cement (al-Mukhtar and Webb, 1971; Coggins, 1973; Heasman et al., 1984; Kloc et al., 2004; Tourte et al., 1981; Wylie et al., 1985). The mitochondrial cement is often found near or in contact with centrioles (Kloc et al., 2004). In addition to mitochondrial cement, the PMC contains a small number of germinal granules and a large number of branched accumulations of electron-dense granulofibrillar material (GFM). The GFM was often observed in contact with mitochondrial cement from one side and with germinal granules from the other. These observations resulted in the commonly accepted conclusion that the GFM originates in mitochondrial cement and by itself is a precursor of germinal granules of stage I oocytes (Heasman et al., 1984; reviewed in Kloc et al., 2001a). These processes are summarized in Fig. 2.
B. Molecular Composition

The composition of nuage and mitochondrial cement in *Xenopus* is, so far, a complete mystery. Because it is believed that mitochondrial cement originates in the nucleus it is plausible that it contains RNAs and/or proteins that shuttle between the nucleus and cytoplasm. Also, because it is believed that mitochondrial cement is a precursor of GFM and ultimately the germinal granules, it may contain structural or regulatory molecules that are needed for the formation and/or function of the latter. However, any speculation on the possible composition and function of mitochondrial cement should await further studies.

Various RNAs and proteins have been discovered localized in the mitochondrial cloud and in the germ plasm in *Xenopus* oocytes and embryos.

C. RNAs

There are two major pathways of RNA localization in *Xenopus*: (1) METRO or early pathway-localizing RNAs such as Xcat2, Xdazl, Xpat, Xlsirts, Xwnt111, DEADSouth, Fingers, XFACS, and Xtox1. These RNAs are present in the MC in early stage I oocytes and anchor as a distinct disk at the tip of the vegetal pole in stage VI oocytes (Chan et al., 2001; Forristall et al., 1995; Zearfoss et al., 2004); (2) Late or Vg1 pathway-localizing RNAs, such as Vg1 or VegT, do not use the MC for their localization (reviewed in Kloc et al., 2001a). A pathway that is a combination of the METRO and Late (Vg1-like) pathways has been described for RNAs such as fatvg and Hermes. These RNAs enter the MC in late stage I oocytes and anchor in the large area of the vegetal cortex in stage VI oocytes (Forristall et al., 1995; Kloc and Etkin, 1995; reviewed in Kloc et al., 2001a). The localization of METRO pathway RNAs within the MC and the germ plasm islands in the embryo has been studied in great detail (Kloc et al., 2002). Xcat2 encodes the Nanos-like zing finger protein (Forristall et al., 1995). It is localized in the METRO region of the MC and later in the germ plasm islands in oocytes and embryos. Electron microscopy *in situ* hybridization showed that Xcat2 is localized in the GFMs and subsequently in germinal granules in oocytes and embryos, and it is released (or degraded) from the granules around the neurula stage of development (Forristall et al., 1995; Kloc et al., 2002). Xdazl encodes an RNA-binding protein homologous to DAZ-related genes that are involved in germ cell development (Houston et al., 1998). It is present in GFM in prestage I and stage I oocytes. In older oocytes and embryos, Xdazl is present in the cytoplasm between, but not in, the germinal granules (Kloc et al., 2002). Xpat encodes a novel protein (Hudson and Woodland, 1998). It is present in GFM and on the periphery of germinal granules in
Xlsirts, a noncoding RNA, belongs to a family of highly repetitive RNAs (Kloc et al., 1993; Zearfoss et al., 2003). It is present in the center of the PMC in oogonia and in the cytoplasm between, but never in, the germinal granules (Kloc et al., 2002). Xwnt11 encodes a signaling protein from the wnt family (Ku and Melton, 1993). It is present in oocytes and embryos between, but never in, the germinal granules (Kloc et al., 2002). DEADSouth encodes DEAD-box Vasa-related RNA helicase. Closely related to eIF4A (MacArthur et al., 2000), it is present in GFM and at low levels on the periphery of germinal granules in stage I oocytes. In embryos it is present between, but not on, the germinal granules (Kloc et al., 2002). Fingers encodes a protein containing a Kruppel-like zinc finger motif and is homologous to the Kox1 protein of transcriptional repressors. It is not detected after fertilization, and is never present on germinal granules (Kloc et al., 2002). XFACS encodes a long-chain fatty acyl-CoA synthetase or ligase, and is 75% identical to mouse FACS. It is never detected in germinal granules (Kloc et al., 2002). Xtox1 is the Xenopus homolog of vertebrate Otx and the orthodenticle (otd) gene of Drosophila (Pannese et al., 2000). Its localization in germ plasm in embryos and ultrastructural localization in oocytes and embryos has not been studied. Two known RNAs that localize through the combination of METRO and Late pathways are FatVg, which encodes the Xenopus homolog of mammalian adipose differentiation factor (Chan et al., 2001) and that is present in the MC of oocytes and in germ plasm islands of embryos but has never been observed inside the germinal granules (Kloc et al., 2002), and Hermes, which encodes an RNA-binding protein (Gerber et al., 1999; Zearfoss et al., 2004) and is present in the MC of oocytes but whose ultrastructural localization has not been studied.

The interesting question is how all these RNAs enter the MC and/or germinal granules and how they are anchored at their destinations. Passive diffusion and simple trapping cannot be excluded as a mechanism for the localization of some of these RNAs. It has been shown experimentally, at least in the case of Xlsirts and Xcat2 RNAs, that there are distinct localization elements that direct RNA molecules to the METRO region of the MC or germinal granules (Kloc et al., 1993, 2000; Zhou and King, 1996). It seems that there is also a common consensus signal in various localized RNAs that directs these molecules to the MC and vegetal cortex of the oocyte (Allen et al., 2003; Bubunenko et al., 2002).

Although, with a few exceptions (see below), the role of all these RNAs is unknown. The fact that some of them are localized within, and some outside, the germinal granules, along with the dynamic and transient nature of their localization, suggests differences in their functions. The RNAs that are localized within the germinal granules are probably directly involved in the determination of germ cell fate. Because some of these RNAs encode RNA-binding proteins and regulators of translation, it is possible that their
protein products are involved in the translational regulation of other germ line-specific RNAs located in the germinal granules. On the other hand, the RNAs located outside of the granules may be involved either in the formation or anchoring of the granules or may have functions completely unrelated to germ cell fate.

D. Proteins

Information about the protein composition of the MC in *Xenopus* is limited. There are only three proteins that are known, with certainty, to be present in the MC. One of them is Spectrin, which is abundant in the mitochondrial aggregate, PMC, and MC in oogonia and oocytes and in the germ plasm islands in the embryo but has never been found inside the germinal granules (Kloc et al., 1998). Spectrin is believed to play a structural and/or signaling role (De Cuevas et al., 1996). Another protein is an elongation factor EF-1α, which is present in the MC of previtellogenic oocytes (Viel et al., 1990). There is no information about the ultrastructural localization of this protein or its localization in older oocytes or in the germ plasm of the embryo. It was reported that germinal granules of cleaving *Xenopus* embryos contain the Vasa protein (Watanabe et al., 1992). However, the antibody used in this electron microscopy immunolocalization was made against a synthetic polypeptide containing the DEAD-box sequence of *Drosophila* Vasa. Because there is a whole family of DEAD-box Vasa-like proteins (including DEAD-South) one cannot exclude the possibility that the protein that was localized in the germinal granules in *Xenopus* was in reality not a homolog of *Drosophila* Vasa but another closely related protein from the same Vasa-like family. Although it was reported that the Vasa-like protein XVLG1 is present in the cytoplasm of *Xenopus* oocytes, the protein was found to be excluded from the MC (Ikenishi and Tanaka, 2000). The type IB activin receptor XALK4 was found to be localized in the MC of stage I oocytes and in the vegetal cortex of vitellogenic oocytes. Electron microscopy showed that the distribution of XALK4 was limited to the endoplasmic reticulum present within the MC, and it was suggested that the activin signaling in *Xenopus* might be mediated by its localization in the MC (Fukui et al., 2003).

E. Function

For many decades, the function of the MC has remained a mystery, although there was speculation that it plays a role in the multiplication of mitochondria or in the production of yolk (reviewed in Guraya, 1979). Molecular and ultrastructural studies showed unequivocally that, at least in *Xenopus*, the MC is a vehicle for the transport not only of germinal
granules and a subpopulation of germ line-specific mitochondria but also localized RNAs to the vegetal cortex of the oocyte (reviewed in Houston and King, 2000a; Kloc et al., 2001a). The depletion of one of the RNAs localized in the MC, Xdazl mRNA, resulted in the failure of PGC migration (Houston and King, 2000b). The depletion of the maternal pool of noncoding repetitive RNA Xlsirts in stage VI oocytes showed that it is involved in anchoring the Late pathway-localizing Vg1 RNA to the vegetal cortex of the oocytes (Kloc and Etkin, 1994). However, because MC mutants are not available and, at present, it is not feasible to remove all or parts of the MC and monitor oocyte or embryo development, there is no evidence of other possible functions of the MC.

### III. Drosophila melanogaster and Other Insects

Germinal granules present in the oocytes of fruit fly Drosophila and other insects have been called polar granules. The most extensive ultrastructural, molecular, and functional studies have been carried out on polar granules and germ cells of Drosophila melanogaster. In this species, the polar granules become visible first in the midvitellogenic (stage 9) oocyte in the specialized cytoplasm of the posterior pole, termed the pole plasm or ososome (King, 1970; Spradling, 1993). They are composed of patches of electron-dense fibrillar material. Initially, they are free in the pole cytoplasm, but toward the end of oogenesis they become associated with the mitochondria (Mahowald, 2001). Thus they look similar to the germinal granules surrounded by mitochondria in the stage IV–VI oocyte of Xenopus. However, in another species, Drosophila hydei, polar granules do not associate with the mitochondria (Mahowald, 1968). In lower dipterans, such as Miastor and Smittia, the polar granule material does not form individual granules but appears as a network of fibrillar material that never associates with mitochondria (Mahowald, 1975; Zissler and Sander, 1973). After fertilization, the polar granules in Drosophila fragment into smaller units about 250–500 nm in diameter and associate with ribosomes (Mahowald, 2001). Similarly, in Miastor, the mesh of the polar granule material fragments into individual granules (Mahowald, 1975). During early embryogenesis of the fruit fly, the germinal granules segregate to the pole cells, and during pole cell divisions they aggregate around centrioles (Counce, 1963; Mahowald, 1962, 1968). Eventually, after the completion of divisions, they fuse into large (approximately 1 μm in diameter) granules. In Drosophila immigrans, all granules aggregate into a single 2- to 3-μm structure (Mahowald, 1968). During the migration of the pole cells toward the gonad in late embryogenesis, polar granules disappear. In PGCs, like in Xenopus, they are replaced by the nuage that appears in the vicinity of the nuclear envelope (Mahowald,
1971). The formation of the polar granules and the cycle of their aggregation and dispersal and even the dimensions of the granules and granule aggregates in *Drosophila* are similar to those in *Xenopus* and *C. elegans*.

Ovaries of *Drosophila* and those of some other holometabolous insects are meroistic–polytrophic. In this ovary type, each developing oocyte remains connected by intercellular bridges (also termed ring canals) to polyploid nurse cells. In *Drosophila* the origin of polar granules in oocytes can be traced to the nurse cells. Because the oocyte nucleus is transcriptionally quiescent, the nurse cells produce and deliver massive amounts of nutrients (RNAs, proteins, and organelles) to the oocyte cytoplasm. The nurse cells also produce electron-dense nuage which first accumulates in the vicinity of their nuclear envelopes and then is transported via the ring canals to the oocyte and becomes incorporated into (or delivers components to) the polar granules (reviewed in Mahowald, 2001). Another novel subcellular component of nuage/polar granule pathway, described in *Drosophila* by Wilsch-Bräuninger et al. (1997), is referred to as the spongelike body. The sponge bodies are present in the cytoplasm of nurse cells and oocytes from stage 3–4 until stage 10–11, and consist of ER-like elements immersed in electron-dense, amorphous material. The latter often contains small aggregations of nuage. Initially (during stages 4–9) the sponge bodies are relatively large and are surrounded by numerous mitochondria. At stages 7–9 they fragment into smaller aggregates that still remain in contact with the mitochondria, but eventually (stage 10) the association with the mitochondria is lost. Concurrently, the sponge bodies are transported from the nurse cells to the oocyte via the ring canals by an unknown (but microtubule-independent) mechanism (Wilsch-Bräuninger et al., 1997). In contrast to nurse cells, the sponge bodies present in the ooplasm are poorly distinguishable and occur as small patches of electron-dense, amorphous material often associated with nuage. Before stage 9 they are dispersed in the oocyte cytoplasm and then at stage 9 (when polar granules form in the oocyte) they are visible in the vicinity of the polar granules (Wilsch-Bräuninger et al., 1997). However, the most recent discovery of true Bbs in oocytes of *Drosophila* (Cox and Spradling, 2003) challenges this opinion and indicates that sponge bodies are not Bbs at all but are nurse cell RNA transport complexes. Cox and Spradling (2003) found that *Drosophila* oocytes in region 3 of the germarium contain typical Bbs composed of clusters of mitochondria, Golgi vesicles, and centrioles. The Bb-derived mitochondria preferentially associate later in oogenesis with the forming germ (pole) plasm.
A. Molecular Composition of Nuage, Polar Granules, Balbiani Bodies, and Sponge Bodies

The molecular composition of pole plasm, polar granules, nuage, and sponge bodies in *Drosophila* has usually been deduced from mutational and functional analyses and indirect genetic approaches (reviewed in Mahowald, 2001). However, several studies showed directly the localization of certain molecules to distinct components of the germ plasm (oosome). At present, four proteins are known to be localized in nuage in *Drosophila* nurse cells and oocytes: Vasa, a DEAD-box RNA helicase with eIF4A homology, implicated in translational control (Hay *et al.*, 1988); Aubergine, a member of the RNA interference (RNAi) defective/Argonaute1 (RDE1) protein family involved in RNAi processes and required for efficient translation of Oskar and thus the pole plasm assembly (Harris and Macdonald, 2001); Tudor, a novel protein implicated in RNA binding (Bardsley *et al.*, 1993); and Maelstrom, a novel protein that localizes to the nuage in a Vasa-dependent manner and is required for proper modification of Vasa and shuttles between the nucleus and cytoplasm in nurse cells (Findley *et al.*, 2003). In oocytes, Maelstrom has diffuse distribution in the cytoplasm and does not show posterior localization in the pole plasm (oosome) (Findley *et al.*, 2003). RNAs and proteins that are known from direct or indirect approach studies to be localized in either pole plasm or polar granules are as follows: Pgc, a noncoding germ line-specific small RNA that was found by electron microscopy (EM) immunostaining in polar granules and is required for pole cell migration (Nakamura *et al.*, 1996); gel (germ cell-less), which encodes nuclear pore protein and is needed for pole cell formation (Jongens *et al.*, 1992, 1994); Nanos, a zinc finger protein required for pole cell migration and the repression of Sxl expression in pole cells (Kobayashi *et al.*, 1996); Oskar, a novel protein required for posterior germ plasm formation (Breitwieser *et al.*, 1996); and Vasa and Tudor, required for polar plasm assembly (Bardsley *et al.*, 1993; Hay *et al.*, 1988). Other RNAs and proteins that are involved in posterior pole formation and thus are indirectly involved in germ plasm formation are listed in other reviews (Houston and King, 2000a; Mahowald, 2001).

At present, the only known molecular components of the sponge body are as follows: Exuperantia, which is required for proper localization of bic mRNA to the anterior pole and of osk mRNA to the posterior pole of the oocyte (Wilhelm *et al.*, 2000); Me31B, DEAD-box RNA helicase and translational silencer (Nakamura *et al.*, 2001); and cold shock protein YPS (Wilhelm *et al.*, 2000). It has been suggested that the nuage–sponge body complex in nurse cells is involved in micro-RNA maturation and/or assembly of ribonucleoprotein (RNP) particles required for proper translation and correct localization of mRNAs in the *Drosophila* germ line (Findley
et al., 2003). Oskar and orb mRNAs and Cup and Orb proteins were found to be temporarily colocalized with the Bb in Drosophila (Cox and Spradling, 2003). This suggests that besides being involved in the transport of germ line-specific mitochondria, Bbs in Drosophila also transport germ line-specific mRNAs and proteins (Cox and Spradling, 2003).

B. Other Insects

For decades, it was commonly accepted that insects did not have discrete and readily recognizable Bbs in oocytes. The only known exception was the presence of Bbs in oocytes of the bedbug, Cimex lectularius (reviewed in Guraya, 1979). Although the accumulations of mitochondria, sometimes associated with nuage material and/or centrioles, have been described in young oocytes of certain insects (the primitive wingless dipluran Catajapyx, beetles, stoneflies, and Plecoptera), they have never been referred to as Bbs (Bilinski and Szklarzewicz, 1992; Jaglarz, 1992; Rosciszewska, 2001) (Fig. 4).

Bradley et al. (2001) reported the presence of Bbs in oocytes of the orthopteran Acheta domesticus (house cricket) and Jaglarz et al. (2003) reported their presence in oocytes of the tiger beetle, Pseudoxycheila angustata (Fig. 4). In Acheta domesticus, there are two roundish Bbs, anterior and posterior, in late previtellogenic oocytes. They contain numerous mitochondria, ER elements, microtubules, and individual Golgi complexes. At the molecular level, they are composed of EF1-α protein, α and γ tubulin, and RNAs with some homology to Xcat2, Xlsirts, and Xpat (Bradley et al., 2001). They are formed in early oogenesis in contact with the oocyte nucleus. The presence of nuage and/or germinal granules has not been reported in Acheta, so the role of Bbs in this species awaits further study. Interestingly, Bbs are present only in the American, not the European, population of Acheta domesticus (Bradley, Bilinski, and Kloc, unpublished). Chang et al. (2002) investigated the formation of germ cells in another orthopteran Schistocerca gregaria (a grasshopper, the African plague locust), using vasa homolog Sgvasa as a molecular marker. Although Vasa RNA and protein were abundant in oogenesis they were not localized to any specific region of the ooplasm. However, in PGCs, the Sgvasa protein accumulated asymmetrically, resembling the asymmetrical position of the germ plasm and nuage in PGCs of other animals (Chang et al., 2002). Much more is known about the possible function of Bbs in the tiger beetle Pseudoxycheila angustata (Jaglarz et al., 2003). In this species, the Bb becomes morphologically distinct in early previtellogenic oocytes and consists of mitochondria, multivesicular bodies (MVBs), and Golgi complexes. As previtellogenesis progresses, the constituents of the Bb become segregated. Mitochondria and Golgi complexes remain in a juxtanuclear position, whereas MVBs are
Figure 4  Balbiani body and oosome in insects. (A) Bb in an early previtellogenic oocyte of the wingless insect *Catajapyx aquilonaris*. Arrow indicates nuage; m, mitochondria. EM micrograph. (B) Bb (long arrow) and oosome (short arrow) in a late previtellogenic oocyte of the tiger beetle *Pseudoxycheila angustata*. n, Nucleus. Semithin section, methylene blue. [From Jaglarz et al. (2003), reprinted with the permission of *Differentiation*.] (C) Oosome (arrow) at the posterior tip of an early vitellogenic oocyte of a parasitic wasp (*Cosmoconus* sp.). Semithin section, methylene blue. Scale bars: (A) 450 nm; (B) 25 μm; (C) 14 μm.
specifically targeted to the posterior pole of the oocyte (the oosome/pole plasm), where MVBs lose their limiting membranes and release their cargo to the surrounding ooplasm. The authors believe that MVB cargo might be involved in the formation of polar granules. Quite surprisingly, striking similarities exist in the morphology and developmental changes of Bbs between *Pseudoxycheila* and *Xenopus*. In both species, the Bb faces the future posterior (vegetal) pole of the oocyte, fragments during late previtellogenesis, and participates in the formation of the germ plasm/oosome. Interestingly, a European species of tiger beetle, the common tiger beetle *Cicindela campestris*, does not have Bbs in its oocytes (Jaglarz et al., 2003; and see discussion below).

### IV. *Caenorhabditis elegans*

Extensive literature covers the germ line specification in the round worm *Caenorhabditis elegans* (reviewed in Seydoux and Schedl, 2001). The P granules that are believed to be a structural and functional equivalent of germinal granules in other organisms are found in germ cells and germ line blastomeres throughout the life cycle of *C. elegans* (Strome and Wood, 1982; reviewed in Seydoux and Schedl, 2001). In oogonia, granules are visible as the irregularly shaped islands of electron-dense material aggregated around the nuclear envelope and traversing nuclear pores. There are, on average, 30 granules per oogonium and 40 granules per oocyte, and the size of the granules increases between the oogonium and oocyte stages. Interestingly, the centrioles were observed at an invariable distance (less than 0.2 \( \mu m \)) from the granules or embedded inside them, and microtubules and mitochondria were often found in close vicinity (Pitt et al., 2000). In oocytes, P granules lose their contact with the nuclear envelope and fragment into smaller particles, and the nuclear pore material becomes included in the P granules. In mature oocytes there are, on average, more than 100 small granules (Pitt et al., 2000). The attributes of P granules in oogonia are highly reminiscent of nuage and germinal granules in *Xenopus* oogonia and prestage I oocytes (see above), and the fragmentation of granules in *C. elegans* oocyte is similar to the fragmentation of *Xenopus* germinal granules in vitellogenic oocytes. However, in contrast to *Xenopus*, the granules of *C. elegans* remain individual entities uniformly distributed in oocyte and zygote cytoplasm. The movement of P granules after fertilization depends on the general flow of cytoplasm toward the posterior pole. However, the association of the granules with the posterior cortex and their segregation during cleavages depend on microtubules, microfilaments, and the product of the gene *mes-1* (Hird et al., 1996; Strome et al., 1995). Numerous studies have analyzed the molecular composition and function of P granules in wild-type and mutant
C. elegans (reviewed in Houston and King, 2000; Seydoux and Schedl, 2001). It seems that, as in Xenopus, the RNA and protein components of P granules change with the stage of the C. elegans life cycle. Proteins such as PGL-1, GLH-1, GLH-2, GLH-3, and GLH-4 are implicated in the regulation of translation and are found in P granules at all stages of the C. elegans life cycle (Draper et al., 1996; Gruidl et al., 1996; Guedes and Priess, 1997; and reviewed in Houston and King, 2000a; Mello et al., 1996; Seydoux and Schedl, 2001). However, proteins such as GLD-1, PIE-1, MEX-1, and POS-1 are present only on P granules in early stages of the embryo (Draper et al., 1996; Guedes and Priess, 1997; Mello et al., 1996; Tabara et al., 1999). GLD-1 is a translational regulator (Jan et al., 1999). PIE-1, MEx-1, and POS-1 are required for the acquisition of germ line fate (reviewed in Houston and King, 2000a; Seydoux and Schedl, 2001), and MEX-3 prevents somatic blastomeres from entering germ line fate (Draper et al., 1996). All of these diverse proteins have potential RNA-binding domain motifs suggesting that the P granules might be a focus of RNA–protein interactions (reviewed in Houston and King, 2000a; Seydoux and Schedl, 2001). Among various RNAs mex-1, gld-1, and nos-2 (similar to Drosophila nanos) mRNAs were found in the P granules of blastomeres but not in the P granules of oocytes (Schisa et al., 2001; Subramaniam and Seydoux, 1999). Interestingly, similar to the germinal granules in Xenopus, the P granules of C. elegans oocytes contain proteins that belong to the family of DEAD-box helicases with homology to Drosophila Vasa, such as GLH and PGL-1 (Gruidl et al., 1996; Kawasaki et al., 1998).

V. Planarians

Planarians are free-living carnivorous flatworms with an extraordinary capability for regeneration that arises from the distinct subset of their cells called neoblasts (Baguñà, 1981). Neoblasts contain a characteristic structure known as the chromatoid body (sometimes also called a nucleolus-like body), which has been compared to germinal granules or Bbs (Coward, 1974; Hori, 1982). The chromatoid body is present in undifferentiated neoblasts, but it disappears from fully differentiated somatic cells that form from neoblasts during the regeneration process. Interestingly, the germ cells that also differentiate from the neoblasts retain the chromatoid body (Hori, 1982, 1992). These observations suggest that the presence of the chromatoid body in neoblasts and germ cells is related to the state of pluripotency and totipotency (Coward, 1974). Ultrastructural analysis showed that the chromatoid body forms by fusion of patches of electron-dense material (nuage) that emanates from the nucleus through the nuclear pores. After moving away from the nucleus, this material associates with mitochondria (Coward,
1974; Hori, 1982). This process resembles the formation of mitochondrial cement and germinal granules in *Xenopus* and P granules in *C. elegans*. The Vasa-like gene products that are present in germinal granules in various animals have also been isolated from planarian *Dugesia japonica* (Shibata *et al*., 1999). In regenerating planarian DjvlgA, RNA was found concentrated in neoblasts and germ line cells, but the exact subcellular localization of the RNA has not been studied (Shibata *et al*., 1999). Because the cells expressing DjvlgA also contain the chromatoid body, the authors suggested the possibility that the chromatoid body in planarians is the functional equivalent of germ plasm and germinal granules in other animals (Shibata *et al*., 1999).

VI. Chaetognaths

Chaetognaths (arrow worms) are peculiar marine benthic and planktonic organisms that taxonomically are situated between protostomia and deuterostomia (Giribet *et al*., 2000). In 1909, Elpatievsky noted the presence of a unique cytoplasmic body in the fertilized egg of *Sagitta*. Subsequently, Wilson in 1925 and Ghirardelli in 1968 suggested that this cytoplasmic body might represent a germ cell determinant in chaetognaths. Amazingly, it took nearly a century to describe the ultrastructure and detailed behavior and identify the first molecular component of the germ granule in chaetognaths (Carré *et al*., 2002). The authors studied the germ granule in two species of chaetognaths, *Spadella* and *Sagitta*. They called it interchangeably germ granule/germ plasm/nuage. They found that, in PGCs the ring of electron-dense nuage material is located around the nucleus and in contact with the more peripherally located ring of mitochondria. In oocytes, nuage material is visible in patches on both sides of the nuclear envelope. During maturation of the oocyte and subsequent fertilization, this material fragments into smaller granules (300–500 nm in diameter) that translocate vegetally by an unknown mechanism. Just before the first cleavage, small granules aggregate into a large (1500 nm in diameter) germ granule that is inherited until the 32-cell stage by a single vegetal blastomere. At the 64-cell stage, the germ granule splits between two vegetal blastomeres and ultimately is inherited by four PGCs. The authors found that, through all these stages, the germ granule/nuage material contains Vasa protein as shown by positive staining with *Drosophila* anti-Vasa antibody. They suggested that there is a general similarity between the germ granule in chaetognaths and the germ plasm in insects, worms, and vertebrates (Carré *et al*., 2002). We postulate that there is an extraordinary resemblance between the behavior and ultrastructure of the germ granule in chaetognaths and the nuage, mitochondrial cement, and germinal granules in *Xenopus*. The chaetognath nuage in the vicinity of the
nuclear envelope and in contact with mitochondria is reminiscent of nuage and mitochondrial cement in *Xenopus*. The translocation of the granules to the vegetal cortex, the cycle of dispersion and aggregation of germ granules, and even the size of granules and their behavior during the cleavage, are all nearly identical to those in *Xenopus*. It seems that the major difference is that in chaetognaths, as in *C. elegans*, the germinal granules lie free in the cytoplasm and translocate to the vegetal cortex as individual entities, whereas in *Xenopus* they are encased within the MC and translocate within its fragments. In addition, there is only one large germ granule in chaetognaths and about 80 granules per embryo in *Xenopus*. It will be extremely interesting to see what other dissimilarities and similarities exist at the molecular level between the mitochondrial cement and the germinal granules in *Xenopus* and chaetognaths.

VII. Ascidians

It has been reported that early embryos of ascidians contain a characteristic structure called the centrosome-attracting body, or CAB, which ultrastructurally corresponds to the germ plasm of *Xenopus, C. elegans*, and *Drosophila* (Hibino et al., 1998). The cleavage pattern in an ascidian embryo is unique. The fourth, fifth, and sixth cleavages are unequal, producing smaller posterior blastomeres. The occurrence of unequal cleavages depends on the function of the CAB. In cleavage interphases, the microtubules emanating from one of the two centrosomes attach to the CAB. Subsequently, the microtubules shorten, leading to the eccentric positioning of the mitotic pole and resulting in unequal cleavage (Conklin, 1905; Hibino et al., 1998; Satoh, 1979). At the ultrastructural level, CABs are recognizable as electron-dense cytoplasmic masses in the posterior tips of most posterior blastomeres. Mitochondria were found around, but not within, these masses (Iseto and Nishida, 1999). The CAB has regions of low, medium, and high electron density. Small granules resembling ribosomes, ER-like membranes, and vesicles are the most abundant in low- and medium-density regions (Iseto and Nishida, 1999). After the sixth cleavage, CABs detach from the cortices and remain in posterior blastomeres (B7.6 pair) that do not divide further during embryogenesis (Iseto and Nishida, 1999). The precursors of the CAB are first recognizable as numerous granules at the posterior–vegetal cortex in a two-cell embryo (Hibino et al., 1998). The granules gradually aggregate, and in the late four-cell stage they form elongated islands on both sides of the cleavage furrow (Hibino et al., 1998). The only known molecular component of the CAB is kinesin-like protein, which may play a role in the attraction of microtubules to the CAB (Nishikata et al., 1999). In ascidians, two different pathways for RNA localization have been identified along with
the cis elements directing the localization of maternal mRNAs to the posterior pole of the embryo (Sasakura and Makabe, 2002; Sasakura et al., 2000). However, the origin of the germ line and the fate of posterior blastomeres in ascidians are so far unknown, and it has been suggested on the basis of findings discussed above that the CAB is the ascidian equivalent of germ plasm (Iseto and Nishida, 1999). A homolog of the vasa gene called Ci-DEAD1 was cloned from Ciona intestinalis (Fujimura and Takamura, 2000). Transcripts of this gene were observed in the most posterior blastomeres (B7.6, containing the CAB) and later in the gonad rudiment and in the germ cells. On the basis of this finding, the authors suggested that B7.6 blastomeres represent a precursor of primordial germ cells in ascidians (Fujimura and Takamura, 2000).

VIII. Urodeles

It is believed that in urodeles the germ cells develop by induction from the pool of totipotent precursor cells in the marginal zone of the embryo without the participation of germ cell fate determinants (Humphrey, 1929; Ikenishi and Nieuwkoop, 1978; Smith, 1964). Despite this, a material resembling nuage was found in the equatorial region of the axolotl egg and in PGCs in embryos (Ikenishi and Nieuwkoop, 1978; Williams and Smith, 1971). However, the fact that the differentiation of PGCs in urodeles occurs much earlier than the appearance of the nuage suggests indirectly that the latter does not play a role in determining germ cell fate (Ikenishi and Nieuwkoop, 1978). Johnson et al. (2001) cloned an Axdazl-axolotl homolog of Xdazl that is known to be present in germinal granules in Xenopus. By studying the localization pattern of Axdazl, the authors tried to resolve the controversial issue of germ cell determination in urodeles. They found that, although Axdazl is specifically expressed in both male and female gonad, it is not localized to any specific structures in oocytes. This supports the hypothesis that axolotls (and urodeles in general) do not have germ cell fate determinants (Wakahara, 1996)

IX. Zebrafish and Other Teleosts

Over the years, the zebrafish Brachydanio rerio (Danio rerio) has become popular as a model organism for biological research. Ultrastructural studies showed that oogonia and previtellogenic oocytes (stage IB primary growth oocytes) of zebrafish contain granulofibrillar nuage in close proximity to the nuclear envelope (Selman et al., 1993). Whenever the nuage was found surrounded by mitochondria, it was referred to as intermitochondrial
cement (Selman et al., 1993). The presence of intermitochondrial cement was also reported for another member of the Cyprinidae family, tench (Tinca tinca) (Clerot et al., 1977). The nuage and mitochondrial cement in zebrafish and tench resemble the corresponding structures in oocytes of Xenopus, C. elegans, and germ cells of Drosophila. In zebrafish, the nuage and mitochondrial cement disappear during oocyte growth and are no longer visible in vitellogenic oocytes. The presence of Bbs was never reported in zebrafish oocytes. However, during embryogenesis, nuage-like accumulations have been reported to segregate to a subset of blastomeres and ultimately into PGCs (Knaut et al., 2000). In fertilized eggs the nuage-particles (1 μm in diameter) were found in the vicinity of the actin cortex. During first division, they aggregated into larger complexes that remained in close contact with microtubules and mitochondria. In blastomeres of the four-cell embryo, patches of nuage were visible in the vicinity of the cleavage furrow (Knaut et al., 2000). The pattern of various mRNAs localizing in zebrafish oocytes and the distribution of RNA and protein of their Vasa homolog were studied in oogenesis and embryogenesis (Bally-Cuif et al., 1998; Howley and Ho, 2000; Knaut et al., 2000; Köprunner et al., 2001). One of these was Nanos-like (Nanos 1) mRNA. It was found localized in the germ plasm and PGCs, and Nanos protein was found to be necessary for the proper migration and survival of PGCs (Köprunner et al., 2001). Another was a zebrafish homolog of Vasa. In stage I oocytes, Vasa mRNA was uniformly distributed in the cytoplasm, but Vasa protein was localized into patches (probably representing nuage) around the nucleus. This pattern of localization disappeared during later stages of oocyte growth (Knaut et al., 2000). During embryogenesis, vasa RNA but not Vasa protein was found in the germ plasm islands of four-cell embryos (Knaut et al., 2000). Thus, it seems that, as in other organisms, vasa in zebrafish is a marker of nuage in oocytes and of germ plasm islands in the embryo.

An analysis of the literature indicates that in contrast to zebrafish, most teleost species contain, besides nuage and mitochondrial cement, distinct and easily recognizable Bbs that, like Bbs in Xenopus, fragments and migrates toward the vegetal pole of the oocyte (Fig. 5; Beams and Kessel, 1973; reviewed by Guraya, 1979; Kobayashi and Iwamatsu, 2000; Romagosa et al., 2002). However, because the zebrafish has become a dominant research model, no molecular studies have been conducted on other fish species for years. Thus, important questions remain concerning the extent to which observations on oogenesis and embryogenesis of zebrafish may be assumed for other fishes. As with other model organisms (Drosophila, Xenopus, and mouse) one should be careful in transposing and generalizing the information obtained from the study of individual species to the whole taxonomic group.
Figure 5 Balbiani body in vertebrates. (A) Accumulation of nuage material (arrow) and mitochondria (m) in the perinuclear cytoplasm of an oocyte of tench *Tinca tinca*. EM micrograph (courtesy of Dr. J. Pimpicka, University of Olsztyn). (B) Bb in a midprevitellogenic oocyte of a sturgeon, *Acipenser sturio*. Semithin section, methylene blue (courtesy of Dr. J. Kilarski, Jagiellonian University). (C and D) Bb (arrows) in oocytes of goat (*Capra* sp.).
X. Birds

Numerous earlier studies showed that oocytes in various bird species contain prominent Bbs (Carlson et al., 1996; reviewed by Guraya, 1979). In stage I oocytes, the Bb is visible as a paranuclear aggregate that looks similar to the Bb in stage I oocytes of *Xenopus*. During oocyte growth, the Bb disperses, and its components move to the cortex of the oocyte (Carlson et al., 1996; Chalana and Guraya, 1979). At the ultrastructural level, it is composed of mitochondria, ER, Golgi complexes, and membrane-bound multivesicular bodies (MVBs) similar to MVB observed in oocytes of the tiger beetle (Carlson et al., 1996; Jaglarz et al., 2003). Tsunekawa et al. (2000) isolated a chicken Vasa homolog and observed its germline-specific expression. They found CVH protein in the Bb of oocytes, in cytoplasmic islands located on both sides of the cleavage furrow in the embryo, and in PGCs. In addition, they showed that, like in *Xenopus*, the Bb in chickens also contains spectrin and mitochondria (Tsunekawa et al., 2000). Comparison of Bb ultrastructure, behavior during development, and molecular composition clearly showed that the Bb of birds and *Xenopus* are homologous. Although it seems that the Bb in birds does not contain germinal granules and/or nuage, it has numerous MVBs, the cargo of which, as in insects, might contain and deliver germ plasm components to the oocyte cortex.

XI. Mouse and Other Mammals

For decades, the prevailing view has been that, like those of the urodele amphibians, mammalian oocytes are nonpolar and do not have any structures corresponding to the germ plasm, and the germ cells develop by a combination of induction and interaction events (reviewed by de Smedt et al., 2000). This belief has primarily arisen from the observation of oogenesis and development in the mouse, which is a model organism dominating mammalian studies. However, a careful analysis of old and new literature reveals that what is true for the mouse is not necessarily true for most other mammals. The oocytes of most marsupial mammals and numerous species of eutherian mammals, such as rats, hamsters, guinea pigs, rabbits, goats, buffalo, nonhuman primates, and humans, have distinct polarity and contain Bbs and/or nuage/mitochondrial cement that are similar in ultrastructure and behavior to those in *Xenopus* (Fig. 4; reviewed by de Smedt et al., 2000).
2000, and Guraya, 1979; Kress, 1985; Young et al., 1999). Unfortunately, because no information about the molecular composition of these structures is available, their function and possible homology to Bbs or germ cell determinants in *Xenopus* and other organisms await further study. Ironically, studies on the expression and localization of MVH, a Vasa homolog in the mouse, may shed some light on some of these questions. Toyooka et al. (2000) found that MVH protein was present in mouse germ cells. Although the MVH protein in oocytes was uniformly distributed in the cytoplasm, MVH in spermatocytes was specifically localized to the perinuclear chromatoid body (Noce et al., 2001; Toyooka et al., 2000). The MVH protein was also localized in the chromatoid body of brush-tailed possums, pigs, cows, Japanese monkeys, and humans (Toyooka et al., 2000). The chromatoid body present in male germ cells in many animal species and in neoblasts and germ cells in planarians (see above) is believed to be an equivalent of the germ cell determinant. It forms in proximity to the nucleus and, at the ultrastructural level, resembles the nuage or germinal granules of *Xenopus*, *Drosophila*, and *C. elegans* (Eddy, 1975; Fawcett et al., 1970; Figueroa and Burzio, 1998). Hübner et al. (2003) were able to develop oocytes from mouse embryonic stem cells. This suggests that embryonic stem cells in mice are totipotent and contain all necessary components for the determination of germ cell fate. It will be interesting to see whether the embryonic stem cells in mice contain chromatoid body similar to that present in totipotent cells in planarians. In addition to MVH, the mouse germ cell-specific RNA-binding protein p48/52 homolog to the *Xenopus* oocyte translational regulator RNA-binding protein was also found in the chromatoid body of mouse spermatocytes (Oko et al., 1996). A logical future approach would be to determine whether the proteins and RNAs that are localized in Bbs and germinal granules in *Xenopus*, *Drosophila*, and *C. elegans* are also present in the chromatoid bodies and Bbs of various mammalian species and, ultimately, the function of these organelles in mammals.

**XII. Similarities among Different Organisms**

Although the structure and behavior of germ line-specific structures show extraordinary variability not only among the various taxa but also among different species belonging to the same family, there are also striking similarities and common themes even among evolutionarily distant organisms. In most species, the germinal granules commence as electron-dense patches of nuage docked at the nuclear envelope. In many organisms, the nuage is visible traversing the nuclear pores, so there is high probability that all or some of the nuage components originate in the nucleus or shuttle between the nucleus and nuage. The discovery of the presence of *maelstrom* (a potential
micro-RNA/RNAi pathway gene) RNA and protein in *Drosophila* nuage strongly supports this notion (Findley et al., 2003). At a certain point in their ontogeny, the nuage and/or germinal granules become surrounded by mitochondria. This suggests either the exchange of components between these organelles or delivery from mitochondria of the energy needed for the movement (reviewed in Guraya, 1979; Reunov et al., 2000). Although the presence of mitochondrial ribosomes in germinal granules of *Drosophila* and chromatoid bodies in planarians has been shown (Kobayashi and Okada, 1989; Kobayashi et al., 1993; Sato et al., 2001), their presence in the germinal granules of *Xenopus* reported by Kobayashi et al. (1998) has not been corroborated by Kloc et al. (2001b) and still remains controversial.

The close contact between the centrioles and nuage/germinal granules found in many different organisms suggests that centrioles (or microtubules nucleated by centrioles) play a role in the spatial organization of germ plasm components (Kloc et al., 2004; reviewed in Mahowald, 2001; Pitt et al., 2000). Although some of the components of germinal granules are highly dynamic, the Vasa protein and/or RNA and a set of RNA-binding proteins and translational regulators are common and invariable components of germinal granules in many organisms (Table I). The germinal granules undergo a cycle of aggregation/dispersion/aggregation during their movement among the oocyte cytoplasm, oocyte cortex, and germ plasm islands of cleaving embryo. Once in the embryo, the germinal granules segregate to the subset of blastomeres and eventually to the PGCs. Kloc et al. (2002) suggested that the aggregation/dispersion cycle is necessary for the proper transport and anchoring of the granules at the cortex and for their proper segregation during the cleavages.

It seems that various organisms employ one of two distinct methods to transport germinal granules to the oocyte cortex. In organisms such as *C. elegans* or chaetognaths, the germinal granules lie and move throughout the cytoplasm as separate entities. In organisms such as *Xenopus*, the germinal granules are enclosed within the transporting organelle, the Bb that moves them in unison to their final destination at the oocyte cortex. It is possible that the transport of germinal granule components to the oocyte pole within the MVB, such as in the tiger beetle and birds, may be a variation of the second method. Others (Cox and Spradling, 2003) and we suggest that the Bb is responsible for the accumulation/sorting/maturation of macromolecules (mRNAs) and organelles (such as germ line-specific mitochondria and germinal granules) that are involved in the specification of a germ line. Subsequently, the constituents of the Bb are directed to the posterior (vegetal) pole of the oocyte, where they participate in the formation of the germ plasm.

The question remains why some organisms apply the first method and some the second. We speculate that the appearance, size, and persistence
### Table I Molecular Composition of Nuage and Germinal Granules in Invertebrates and Vertebrates

<table>
<thead>
<tr>
<th>Animal</th>
<th>Nuage/cement</th>
<th>GFM/germinal granules/polar granules</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Xenopus</strong></td>
<td>Unknown</td>
<td>Xdazl mRNA in GFM only; DEAD-South mRNA in GFM and in germinal granules in stage oocytes; Xcat2 mRNA, Xpat mRNA, present in GFM and in germinal granules in oocytes and embryos <em>(Kloc et al., 2002)</em>. Vasa protein in germinal granules in cleaving embryo <em>(Watanabe et al., 1992)</em></td>
</tr>
<tr>
<td><strong>Caenorhabditis elegans</strong></td>
<td>PGL-1, GLH-1–GLH-4 in P granules in all stages of life cycle <em>(Draper et al., 1996; Gruidl et al., 1996; Guedes and Priess, 1997; Mello et al., 1996)</em></td>
<td>PGL-1, GLH-1–GLH-4 in P granules in all stages of life cycle <em>(Draper et al., 1996; Gruidl et al., 1996; Guedes and Priess, 1997; Mello et al., 1996)</em>; GLD-1, PIE-1, MEX-1, MEX-3, POS-1, in P granules in early embryo <em>(Draper et al., 1996; Guedes and Priess, 1997; Mello et al., 1996; Tabara et al., 1999)</em></td>
</tr>
<tr>
<td><strong>Planarians</strong></td>
<td>Unknown</td>
<td>Unknown&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Chaetognaths</strong></td>
<td>Vasa <em>(Carré et al., 2002)</em></td>
<td>Vasa <em>(Carré et al., 2002)</em></td>
</tr>
<tr>
<td><strong>Ascidians</strong></td>
<td>Unknown</td>
<td>Unknown&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Urodeles</strong></td>
<td>Unknown</td>
<td>Unknown&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Zebrafish</strong></td>
<td>Unknown</td>
<td>Unknown&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Birds</strong></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Mammals</strong></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

<sup>a</sup>As shown by direct biochemical or microscopy analysis. <sup>b</sup>DjvlgA (Vasa-like) is present in germ cells and neoblasts, but its subcellular localization is unknown *(Shibata et al., 1999)*. <sup>c</sup>CiDEAD1 (Vasa homolog) is present in germ cells, but its subcellular localization is unknown *(Fujimura and Takamura, 2000)*. <sup>d</sup>Axdazl is present in oocytes, but is not localized to any specific structures *(Johnson et al., 2001)*. <sup>e</sup>Vasa was found in germ plasm islands in embryos, but its subcellular localization is unknown *(Knaut et al., 2000)*.
of the Bb are closely related to the ovary type and/or the length of oogenesis (the rate of egg production). It is possible that when oogenesis lasts for a long time the germ plasm components must be protected from uncontrollable scattering throughout the cytoplasm and become enclosed in the aggregation organelle (Bb), and within this organelle they move to the oocyte cortex. In organisms with short oogenesis, the formation and transport of germ plasm components occur so rapidly that there is not enough time or need for the formation of a pronounced Bb, and the germinal granules persist and move as individual entities. In the meroistic-polytrophic ovaries of holometabolous insects (e.g., *Drosophila*), the oocytes are associated with polyploid nurse cells, and this leads to the acceleration of oogenesis. In such ovaries, Bbs, as a rule, should be either absent or, as in *Drosophila*, inconspicuous and short-lived. The oocytes of birds, amphibians, fishes, myriapods, and spiders, which are not associated with supporting cells and develop relatively slowly, should contain large and prominent Bbs. We believe, in this context, that the oocytes of hemimetabolous insects (devoid of nurse cells) likely have Bbs. The occurrence of well-defined Bbs in the oocytes of *Acheta* and the existence of mitochondrial aggregates in the ooplasm of such insects as wingless diplurans and stoneflies support this hypothesis.

In *Drosophila* and *Xenopus*, only a subset of oocyte mitochondria (i.e., Bb-derived mitochondria) becomes preferentially included in the germ plasm. This is especially evident in *Xenopus*, where the MC-derived mitochondria have distinct morphology and stop replicating much earlier than the rest of the oocyte mitochondria (D’Herde *et al*., 1995; Mignotte *et al*., 1987; Tourte *et al*., 1981, 1984). In *Drosophila*, the nurse cell-derived mitochondria are blocked from entering the oocyte through the ring canals during germ plasm assembly; thus, only Bb-derived mitochondria participate in the formation of the germ plasm (Cox and Spradling, 2003). There is evidence that mitochondria accumulate mutations and that, at least in mammals, the removal of defective mitochondrial genomes may play a role in germ line apoptosis (reviewed in Partridge and Gems, 2002; Perez *et al*., 2000). Cox and Spradling (2003) postulated that if only “healthy” mitochondria selectively associated with the Bb, this would be a mechanism for organelle-based selection and the elimination of defective mitochondrial genomes from the ultimate germ cells. The substantiation of this hypothesis awaits further study. However, the discovery of Bbs in *Drosophila*, which is a genetically verifiable model organism, offers a promise of swift progress in decoding this and other functions of the Bb.
Acknowledgments

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References


1. The Balbiani Body and Germ Cell Determinants


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Fetal–Maternal Interactions: Prenatal Psychobiological Precursors to Adaptive Infant Development

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I. Introduction

The issue of why sensitivity to experience became a part of the developmental process for some organisms is an interesting one that merits a more detailed consideration. 

Greenough, 1991, p. 16

The burgeoning of new technologies in developmental biology has enabled the creation of organisms resulting from novel developmental pathways. Stem cells, gene therapies, and reproductive technologies have the potential to maintain or induce developmental plasticity at novel developmental stages. At the organismic level, scientists interested in development and biobehavioral phenomena need to evaluate both the intended and unintended long-term effects of these technologies on infant development. A theoretical framework is being established to assist this research endeavor in the search for genetic, prenatal,

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and postnatal interactions, and to interpret the information produced. This framework needs to have intergenerational adaptation and adaptive behavior as its goal.

With adaptive behavior in mind, processes that produce individual differences will be central to the ideas presented in this chapter. The goal of the first part of the chapter is to summarize postnatal early rearing literature on adaptive behavior. Sackett et al. (1999) developed a theoretical explanation of this literature to explain the developmental interaction between organisms and their postnatal environments. Here this work is applied to understanding prenatal development. With the exceptions of the umbilical connection between mother and fetus and the suppression of behavioral coping strategies by the uterus, many developmental processes discussed as postnatal phenomena originate before birth. It is also unlikely that many developmental processes operate independent of any genetic input. Section II discusses the effects of pre- and postnatal environments relative to genetic predispositions, specifically focusing on central serotonergic (5-hydroxytryptamine, 5-HT) functioning and the serotonin transporter gene (5-HTT; Lesch et al., 1996).

In the next section, prenatal stress literature is reviewed and the suggestion is made that the assortment of effects from negative to none and even positive effects may actually be representative of differential development rather than due to methodological or theoretical problems with the literature. The intention is to challenge many of the common ideas, or perhaps common misinterpretations, coming out of the prenatal stress literature that suggest a main effect for prenatal stress, as “bad.” Environmental perturbations of the fetal environment do not necessarily produce negative outcomes (Denenberg et al., 1998; DiPietro et al., 2003; Novak and Sackett, 1996). Instead, a variety of fetal experiences is necessary for typical development. A model is presented to characterize fetal individual differences that may exist as precursors to postnatal developmental individual differences, specifically maternal–infant synchrony and/or infant reactivity (Tronick, 1989). To make this argument, the literature on classical conditioning of young organisms is offered as a potential mechanism by which prenatal variation in individuals and environments may induce or facilitate the canalization of individual differences in postnatal development. From this perspective, pattern recognition and predictability in the prenatal environment classically condition the infant to be sensitive to the immediate environment, establishing the basis for the performance of postnatal adaptive behavior.

Although the intensity of sensory input increases with the birth experience, with the exceptions of cardiovascular and respiratory shifts that occur as the infant transitions from the aquatic environment of the womb to postnatal life, the sensory systems, and biobehavioral systems for responding to the environment, are functioning before birth (Gottlieb, 1971). It is possible
that the birth experience creates discontinuities in sensation and perception, or that hormone and immune changes associated with birth require new and different ideas for how to think of organismic development before birth. However, the argument is made that principles and ideas for explaining the effects of early postnatal experience can be extended into the womb because of developmental continuities across the birth experience.

II. Early Experience and Postnatal Environments

At the beginning of the twentieth century, the failure of infants to thrive in orphanages and hospital environments was egregious, with death rates for children in New York between 35 and 75% (Chapin, 1915; English, 1984). With the discovery of germs and sterile environments, hospitals combatted these numbers by emphasizing isolation for young children to protect them from illnesses. Despite the increased cleanliness that resulted, fatality rates did not begin to drop until physicians, nurses, and hospital personnel began to attend to the emotional and social needs of children in their care (Bakwin, 1942). This transition, and the backlash against it by competing theoretical perspectives, prompted Harry Bakwin of the Children’s Medical Service at Bellevue Hospital in New York to trumpet, “...humanizing the hospital care of infants has not increased the mortality in the wards” (p. 40). In fact, mortality was lowered. This historical life-and-death battle for the lives of young people in hospitals and orphanages emphasizes the critical role of early experience in behavior. Against this backdrop Harlow, Hubel and Weisel, Hebb, and colleagues set out to isolate the components of environmental stimulation that were necessary and sufficient to produce both typical and atypical structure and function in developing organisms.

In infant monkeys, Harlow and colleagues challenged contemporary beliefs that the primary attachment process of infants involved food and/or a psychodynamic forces, defining the need or drive for “contact comfort” as essential to infant development (Harlow and Zimmerman, 1959). Similar to the findings from hospitals and orphanages, infants clearly preferred warm fuzzy mothers or mother substitutes. Subsequently, the necessary and sufficient components of the environment have been pursued. As opposed to the human models, animal models can experimentally manipulate both the amount and timing of exposure to environmental components. Rodent, non-human primate, and other animal models have all been essential to our current level of understanding. For many processes, however, the nonhuman primate model of complex human behaviors involving cognition and emotional repertoires offers the most compelling view of developmental phenomena due to genetic similarity, and the homology of physiological and behavioral response.
systems to social and nonsocial environmental stimuli. In addition, many developmental patterns between nonhuman primates and humans are parallel, particularly early in development.

In contrast to "contact comfort," "isolation rearing syndrome" was also described in rhesus macaques. This syndrome, of extreme withdrawal from the environment and high levels of stereotypic and even self-injurious behavior, resulted from extended periods of environmental deprivation. The results of these studies have been reviewed elsewhere (Capitanio, 1986). The term syndrome denotes the belief that it was a widely distributed response to environmental deprivation. However, this assumption, when tested, fell apart (Sackett et al., 1976, 1981). Even within closely related macaque species, the phenotypic profile is different, varying in the behavioral profile produced and in the degree of recovery that is possible.

Several theories, both biological and psychological, have been proposed to account for reduced functioning due to early environmental deprivation (Sackett, 1970; Sackett et al., 1999). Are the deficits produced structural or functional? Does deprivation rearing result in degeneration of structural and/or chemical brain processes? Or, perhaps structures and functions are experience expectant (Greenough et al., 1987)? Is it possible that infants reared in impoverished environments fail to learn the basic materials of adaptive behavior so that they are unable to function in developmentally more complex environments? Finally, the degree of difference between rearing environments and later behaving environments may be too large to be attempted, instead producing excessive emotional arousal and/or avoidance rather than adaptive behavior.

Holson and Sackett (1984) concluded, from a review of the literature, that there was no evidence for alteration in the ability of animals to perform adaptive behavior. Instead, deprivation rearing alters an organism’s willingness to behave adaptively. Infant monkeys exposed to various levels of impoverished rearing varied in the time and experience necessary to be trained to perform on learning tasks. However, once adapted, no differences existed in the ability to perform simple learning tasks (Harlow et al., 1969; Rowland cited in Sackett, 1970; Sackett et al., 1999). In addition, the deprivation-reared infants were slower to extinguish learned responses, and were slower to learn response suppression, even in the face of a well-learned conditioned stimulus signaling foot shock (Frank et al., 1977). These findings argue against deficits in learning ability, at least for relatively simple tasks, and also suggest unwillingness to inhibit adaptive behaviors once they have been well learned.

In rodent species Hebb attempted “to learn how the functioning of individual neurons and synapses relates to the functions of the whole brain, and to understand the physiological nature of learning, emotion, thinking, or intelligence” (1955, p. 826). In contrast to the privation studies in nonhuman primates that mirrored human health problems in hospitals and orphanages, the rodent literature focuses on how early environments can induce, facilitate,
or maintain structural and/or functional advantages for the offspring (Gottlieb, 1976a,b). Hebb found that rats reared as pets in his home outperformed rats that had remained in standard laboratory caging (1949). Subsequent to this observation, the developmental and biological properties of this finding have been pursued and summarized (see, e.g., Rosenzweig and Bennett, 1996; Sackett et al., 1999; Spatz, 1996).

Rosenzweig and colleagues demonstrated that rats reared under conditions analogous to those of Hebb’s pets, that is, enriched social, visual, and tactile environments, had increased activity, specifically acetylcholinesterase (AChE), and/or tissue in some areas of the brain, specifically the neocortex, relative to rats reared in single bare cages or rats not given extensive learning opportunities (Krech et al., 1956; Rosenzweig et al., 1962). Noncortical areas are also affected, leading to greater contact between glial cells and synapses in the superior colliculus (Jones and Greenough, 1996). Thompson and colleagues found that for classical conditioning, learning-related differences occurred in the hippocampus, but that the hippocampus was not necessarily involved. Further, altered activity in individual neurons was detected for both the cortex and the deep nuclei of the cerebellum, particularly the interpositus nucleus (Lockhart and Moore, 1975; McCormick and Thompson, 1984; Thompson, 1990). Similarly, Hubel and Weisel discovered changes in brain structure in both the lateral geniculate nucleus and the visual cortex after altering postnatal visual experiences in kittens (Hubel and Weisel, 1965a,b; Weisel and Hubel, 1965). They demonstrated that structural and functional changes were not only dependent on environmental inputs, but also on interactions with other systems. Both extensive and limited reviews beyond the scope of this chapter have summarized the often exquisite research that has been performed to isolate the effects of learning and experience on the brain (Rosenzweig, 1996, 1998; Sackett et al., 1999).

III. Early Experience and Genetics

Despite the pivotal role of experience in differentiation, genetic predispositions remain central to any developmental theory of experience. The zeitgeist that description of the human genome is closing a chapter of developmental theory or knowledge is clearly wrong. However, so is the strictly nurturist perspective. Furthermore, behavior genetics studies, seeking to proportion variance into these two categories, underestimate the dynamic nature of development. Still in their infancy are questions concerning how specific genes are manifested in phenotype, how they interact with pre- and postnatal environments, how modifiable their expression is, and whether redundancies in gene products create multiple genetic pathways that can produce homologous phenotypes. The description of the human genome is a formidable table
of contents to a scientific understanding of adaptive functioning that has yet to be written.

As specific genes and gene products are identified, experiments need to be designed to assess the actual developmental interactions that take place and the extent of between- and within-subject variability that different environments can produce from the cellular to the organismic level and beyond. One example of experimenting with a specific gene–environment interaction involves the research on emotional behavior such as impulsivity, aggression, and anxiety and the serotonin transporter gene (Fairbanks et al., 2001; Lesch and Merschdorf, 2000; Lesch et al., 1996). Previously, twin studies had estimated that 40–60% of variance in anxiety-related behavior could be heritable (Plomin et al., 1994). However, with the molecular genetic discovery of the gene sequence responsible for removing 5-HT from the synaptic cleft, more precise experimental data are now being produced. The 5HTT gene is controlled, at least in part, by a polymorphic repetition at the 5′ end of the transcriptional promoter region of the 5HTT gene, labeled 5HTTLPR. There are at least three alleles at this site. In most studies, the long (L) and short (S) versions of the allele are discussed and not the extralong (XL) allele (Delbruck et al., 2001). The short version of the allele is associated with reduced transcriptional efficiency of the 5HTT gene. Studies of impulsivity (Fairbanks et al., 2001), alcohol consumption (Heinz et al., 2003), and social status (Manuck et al., 2003) have demonstrated the effect of differences in this gene on behavior. Other studies have failed to find this link (e.g., see Perisco, 2002).

Studies investigating effects of the 5HTTLPR polymorphism on behavior show that the postnatal environment interacts with the genetic predispositions. Bennett et al. (2002) compared peer-rearing and mother-rearing among infants with different allelic combinations. The length of 5HTTLPR was predictive of cerebrospinal fluid (CSF) 5-HT metabolite levels, but only in the peer-reared group. In addition, Champoux et al. (2002) performed Brazelton neonatal assessments (Brazelton, 1973), adapted for infant monkeys (Schneider et al., 1991), on both mother- and peer-reared infants. Peer-reared infants with the L/S allele had lower orientation scores than their L/L counterparts. There were no differences between infants in the mother-reared group. Further, Barr et al. (2003) found more severe intoxication behavior in L/S juvenile monkeys in the 30 min following intravenous ethanol exposure, but only in peer-reared monkeys. All of these examples from nonhuman primates may indicate a protective function for mother-rearing that blunts the potential for negative behavioral outcomes associated with the 5HTTLPR polymorphism. In humans, individuals with low socioeconomic status had reduced prolactin responsivity following a fenfluramine challenge, but only if they had at least one S allele. In addition, within the L/S and S/S groups, SES and prolactin response were positively related (Manuck et al., 2003).
The role of the prenatal environment in further modifying these gene–environment interactions is unclear. It is possible that the variance attributed to genetic factors in these studies is entirely an effect of the prenatal environment. It is possible that genetic differences in the ability to transport serotonin within the cell exposes fetuses differentially to protective levels of serotonin. Gould (1999) showed that serotonin functioned to enhance or protect neurons from stress-induced loss in brain areas important to learning. Despite the redundancy of neuronal connections during the brain growth spurt, genetic differences in the availability of serotonin may induce differences in learning ability and susceptibility to stress.

IV. Early Experience and Learning

There has been considerable debate about learning and the brain. The emerging picture is one of incredible plasticity and change in response to experience. Although experiments by Weisel and Hubel (1965) found evidence that there were critical periods to developmental plasticity, a growing number of researchers are concluding that plasticity continues well beyond early life (Rutter, 2002), including the growth of new neurons as an adult in both cortical and noncortical areas of the brain (Gould and Gross, 2002; Gould et al., 1999). Changes in the structure and function of the brain depend on the type of experience, the age of the subject, the area of the brain affected, and/or any modifying processes that might function to impair or inhibit learning (Rosenzweig, 1999). In addition, structural changes as a result of learning and experience can be due to deletions as well as additions in the number of neurons and neuronal connections (Rosenzweig, 1998).

The portion of this debate particularly relevant to the issues being discussed in this chapter concerns the exact organismic functions that changes in brain structure and function are responsible for. At the extremes are the competing ideas that altered rearing environments produce changes in the ability to learn (Hebb, 1949), or that changes due to shifts in environmental complexity alter their motivation or willingness to respond. The maze-bright versus maze-dull rat experiments are popularly known to have resulted in the conclusion that what was bred for in the maze-dull rats was a lack of motivation rather than “dullness” (Searle, 1941, 1949). However, Rosenzweig (1998) disputes this claim, using unpublished data from the maze-bright and maze-dull rats from Searle’s graduate adviser Robert Tryon, and his own data. Rosenzweig reports that Tryon used a larger sample size than did Searle, and found a general deficit in learning rather than the published motivational differences. Rosenzweig then tested descendants of the maze-bright and maze-dull rats and concluded a generalized intellectual deficiency across several tests (Rosenzweig, as cited in Rosenzweig, 1998). In contrast,
the nonhuman primate literature on varied rearing experiences suggests that the alterations are neither learning nor motivation (Sackett, 1965b; Sackett et al., 1999). Rather, after habituating to a particular learning task, isolation-reared monkeys outperformed their socially reared controls, demonstrating a lack in neither ability nor motivation.

V. Complexity Dissonance Theory of Early Experience and Development

We have previously offered *complexity dissonance theory* as a developmental explanation of the effects of varying qualities of environment on postnatal development (Sackett et al., 1999). The theory proposes that the complexity of the rearing environment induces a psychological complexity in the individual (Sackett, 1965a). Individuals who are relatively high in psychological complexity are produced by environments containing the following:

1. Multiple stimulatory experiences requiring the infant to respond
2. Perceptual and motor skill training involving multimodal stimulation
3. Both predictable and unpredictable perceptual-motor feedback
4. Control of at least some external events

In 1957, Dember and Earl offered an explanation of the effects of complexity on curiosity, learning, and manipulatory behavior. Our explanation set these same principles into a developmental context (Sackett et al., 1999). Included in the theory is a potential explanation for one of the self-limiting components of the original theory. Previous versions of this theory had failed to be falsifiable when people tried to generate objective definitions of complexity. For any given object, there is both an objective aspect and a subjective aspect to its complexity. Objective properties are easy to quantify; however, the subjective aspects of object complexity are dependent on individual differences in experience, and remained elusive. Our answer to this problem was that environments vary in the modes of energy they generate. These modes of energy impinge on an organism via their sensory systems. Stimuli that activate a greater number of sensory systems in order to be perceived are more complex than stimuli requiring fewer sensory modalities. Some sensory systems are more similar to each other. For example, the links between smell and taste for all wine connoisseurs are well known. However, the links between taste and proprioception may be less direct. The more diverse the sensory systems activated by a stimulus the more complex the stimulus. These multimodal abilities in human infants have been argued to be a critical component of adaptive development in human infants (Meltzoff and Moore, 1997). Their theory complements the ideas put forth here in
that multimodal experience is critical for adaptive behavior, and that these multimodal abilities exist very early in life, perhaps developing prenatally.

Sackett et al. (1999) argue that the effect of differential rearing is to alter the range of complexity in the environment to which an organism is willing to adaptively respond. These differences in experience establish the psychological complexity of the individual. Environmental complexity and psychological complexity combine to form a pacer range, a range of environments to which an organism willingly responds. Early environments rich in variation, complexity, and predictability produce a wide pacer range. Organisms that have been reared in impoverished environments have a very narrow pacer range. Stimuli in excess of the pacer range overwhelm the organism and they withdraw from the environment, regressing to well-learned high-probability behavioral responses similar to the isolation-rearing syndrome described in rhesus macaques. Stimuli below the level of an organism’s pacer range would also result in a maladaptive behavioral repertoire, which may include boredom and disruptive or stereotypic behavior.

There is some experimental evidence that supports this theoretical interpretation. Sackett (1965b) presented infant monkeys reared in different types of environments with objects that vary in the complexity of stimulus components including an immobile steel bar, a T-shaped metal bar that moved in only two dimensions, up and down, and a hanging metal chain with much more complex movements and auditory inputs. Consistent with our theory, organisms that were reared in complete environmental deprivation interacted only with the immovable bar. Animals reared under standard laboratory conditions spent the majority of their time interacting with the T-shaped bar. Monkeys that grew up in the wild, the most complex environment available to a monkey, interacted most with the complex chain stimulus and practically ignored the immovable bar. Therefore, although there is debate about the genetic effects that were bred for with the maze-dull rats, the rearing effects in nonhuman primates appear to be a narrowing of the pacer range, altering the willingness of an organism to respond adaptively (Sackett et al., 1999).

VI. The Effects of Prenatal Experience: Establishing the Pacer Range

Longitudinal prenatal studies offer the opportunity to search out the origins of the pacer range. Can the parameters of postnatal development be extended beyond the birth experience? There is, of course, no reason to think that the processes of environmental and psychological complexity begin only at birth. The primary literature on this subject involves the effects of prenatal psychosocial stress on infant development. The results from this literature
are somewhat ambiguous, running the gamut of negative and null effects (for a review see Lobel, 1994) and a subset of studies even reporting positive effects of prenatal manipulations and stress (Denenberg et al., 1998; DiPietro et al., 2003; Francis et al., 1999).

Whether positive or negative, the specific mechanism by which maternal psychological experience is transmitted to the fetus is unknown. Proposed mechanisms include impairment of uteroplacental blood flow (Morishima et al., 1978), anoxia to the fetus resulting in organ sparing, which then causes asymmetrical development relative to organs not spared (Thornburg, 1991), and alteration of hypothalamic–pituitary–adrenal (HPA) axis glucocorticoid function mediated by placental transfer of hormones (Weinstock, 1997; Zarrow et al., 1970). In addition, alterations of the hypothalamic–pituitary–gonadal axis resulting in abnormally low testosterone levels in males (Ward, 1984), enhanced humoral immune responses (Klein and Rager, 1995), altered endocrine or behavioral profiles, and endocrine-induced alterations of immune function (Coe et al., 1996) have been proposed. In nonhuman primates, the mechanism most studied concerns alterations of the HPA axis. However, the studies designed thus far have involved indirect measurements, which cannot answer acute questions.

Similar to complexity research, experiments on stress and stress phenomena require that both the environmental context of the stress (Selye, 1980) and the response of the individual (Coyne and Lazarus, 1980) be defined and quantified. For the fetus, the simplest model of psychological stress involving the environment is a mother that either does or does not respond to its environment, with the consequence that the fetus also does or does not respond. Although it is possible for the fetus to respond directly to the environment outside the uterus, independent of the mother, for the sake of this illustration let us limit our discussion to maternally mediated responses. Using this characterization, a $2 \times 2$ research design, we can cross the presence or absence of an acute stress response in the mother with the presence or absence of an acute stress response in the fetus, generating four types of fetal responders (Novak, 2002).

The first is traditionally the subject of prenatal stress studies: stress to the mother that produces stress to the fetus. In the rare case that studies actually measure rather than assume acute stress responses, fetuses whose mothers respond to a stressor, but exhibit no stress response themselves, usually would be classified as an error variance. However, these fetuses also may be different from the stressed fetuses in the first example. Using the approach suggested here, these animals become a second experimental group in the analysis, perhaps called resistant, and are no longer part of the error term. Furthermore, fetuses whose mothers do not experience the stressor and do not initiate a stress response themselves make up the proper control group for studies of stress. However, similar to the first two examples there is another,
complementary group that is often overlooked. This group is composed of fetuses that exhibit a stress–response profile even though their mothers did not experience any particular stressor. This group may be a chaotic group, perhaps even pathological. However, it may be that the stress responses in this group vary from what is typical in chronicity, or the organism may be responding to an unmeasured or latent stressor.

From a developmental viewpoint, alternative explanations exist for the four maternal–fetal response classifications, just described. While these patterns of response may represent individual differences in the maternal–fetal relationship, it is also possible that differences among fetuses are due to differences in maturation. For example, until biobehavioral response systems in a fetus are functioning, a maternal response without fetal response is developmentally appropriate because the fetal nervous system lacks both central and peripheral receptors to perform pattern recognition. As fetal response systems mature, it is likely that the stress responses of the fetus will follow the mother’s response. In this case, the maternal stress responsivity functions as scaffolding on which the fetal responsivity develops, and stressed and unstressed classifications become developmentally appropriate rather than response states. Finally, as fetal stress systems become mature and capable of self-regulation, fetal responsivity would be less likely to match maternal reactions across various situations. In this case, chaotic and resistant classifications are developmentally appropriate. Furthermore, in the unstressed condition, no response from either the mother or the fetus is developmentally appropriate in several situations.

Novak (2002, 2004a) used tethering with maternal and fetal catheterization to assess the acute fetal effects of potentially stressful maternal psychological experiences. Heart rate, blood pressure, and heart rate variability in the mother and the fetus show that the fetal response to maternal stressors is rapid in response to some types of environmental challenges. At other times, the mother shows a cardiovascular response but the fetus does not. And at still other times, the fetal cardiovascular parameters change although there is no apparent maternal response. Currently investigations are studying whether individual differences in the degree to which mothers and fetuses are correlated, in response to environmental demands placed on the mother, have long-term implications.

One inference that can be drawn from these data involves the speed of fetal responding to maternal psychological stimuli. Although the rapidity of response observed in the fetus does not preclude changes in the HPA axis or anoxia as the mechanism by which negative psychosocial experiences of the mother affect fetal development, it is evidence that there must be additional mechanisms. The acute fetal response to maternal psychosocial stress responses is too rapid to be mediated by hormones alone. Candidate mechanisms can be any of the fetal sensory systems, all of which are
functioning this late in pregnancy (Gottlieb, 1971); most likely, however, no one mechanism is solely responsible.

Within the ontogeny of a prenatal stress response may exist the origins of infant reactivity and sensitivity to environmental stimulation. It is during this time period that the postnatal pacer range is being established. As the fetal sensory systems develop, environmental input starts as white noise. In essence, pattern recognition becomes the primary capacity of the infant that will enable an organism to survive. This is particularly true when we characterize the response of the fetus relative to the response of the mother. How the fetus responds when the mother is or is not reacting herself, and subsequently how the fetus recovers, may shed light on the origins of mother–infant reciprocity (Tronick, 1989). Until the fetus becomes capable of distinguishing patterns in the sensory, hormonal, and immunological input it receives, it is at the mercy of the mother via the placenta. Although many maternal hormones do not cross the placenta, hormones often induce the placenta to produce hormones that in turn affect the fetus.

A. Fetal Classical Conditioning and the Challenges of Fetal Biobehavioral Research

Using principles from classical conditioning, one can hypothesize a testable developmental model about origins of fetal responsivity to maternal clues and bidirectional infant–environment interactions. Previously, the appearance of the first conditioned reflexes was argued to be a primarily postnatal phenomenon (Kasatkin, 1972). Ray (1932) found auditory stimuli would produce a fetal motor response. However, Sontag and Wallace (1934) were unable to replicate these results. Although variations of this research paradigm by Spelt did replicate Ray’s data, Lipsitt questioned these conclusions by arguing better controls were needed (cited in Kasatkin, 1972). Conditioned stimuli needed to be less related to the unconditioned stimuli and conditioned response to ensure that fetal responding was not due to sensitization. Lipsitt argued that in most fetal studies reporting evidence of fetal classical conditioning unconditioned stimuli were inherently associated with the type of fetal responsivity they came to produce. Given this argument, Kasatkin (1972) acknowledged that the prerequisite tools were probably in place for conditioning, yet fetal classical conditioning was unlikely.

More recently, Smotherman and colleagues have exquisitely demonstrated classical conditioning in late-gestation fetal rat pups involving the endogenous opiate system of the fetal rat (Smotherman, 2002). Classical conditioning was observed within 30 s of reexposure to the conditioned stimulus and continued to occur for up to 90 s. A greater number of pairings between conditioned and unconditioned stimuli resulted in longer durations of
responding. In addition, individuals who show classical conditioning are more likely to later perform a stretch response. This demonstrates that behavioral and experiential differences can result in individual differences due to differential fetal learning. Smotherman (2003) discovered that for this type of classical conditioning, the retention interval of the learned response was 18–21 min, and that extinction would occur within 15 to 25 exposures when the conditioned stimulus and unconditioned stimulus were not coupled.

Access to the fetus for behavioral research presents unique challenges, therefore, additional literature on classical conditioning in the fetus is lacking, with little discussion of the number of trials, their range of fetal response systems that can be conditioned, and the developmental sequence for different types of fetal responding. Furthermore, individual differences resulting from any form of prenatal conditioning have not been systematically explored. In addition, trace conditioning, a form of classical conditioning involving increased time gaps between stimuli that need to be associated (Solomon et al., 1986, 1996), might have particular relevance because of the slow time course for maternal-to-fetal transmission of hormones and immune products. Trace conditioning in the fetus has not been explored. Postnatal research shows trace classical conditioning is involved in learning and memory processes, and associated with the formation of new neurons (Shors et al., 2001). Glucocorticoids disrupt trace conditioning, but the neurotransmitter serotonin appears either to protect or facilitate this same neural growth (Gould, 1999).

The challenges of studying fetal behavior are diverse depending on the question and the species studied (Novak, 2004b). In humans there is a considerable literature about fetal development resulting from studies using ultrasound technologies (Lecanuet et al., 1995). However, many questions about developmental processes require experimental manipulations not possible during human pregnancy. In rodent species, Smotherman and Robinson (1986, 1987, 1988; Robinson and Smotherman, 1995; Smotherman, 2002; Smotherman et al., 1986) developed an exquisite model in which the rat pup can be exteriorized and yet remain physiologically attached to the mother. Using this preparation, fetal behavior has been characterized in response to both tactile and chemical stimuli. However, the ability to generalize from rodent species to human species is limited, and therefore nonhuman primate models of prenatal development are essential in order to answer many questions of interest to science and human health.

Exact replication of human fetal developmental data is not possible for nonhuman primates. Ultrasound requires anesthetization or potentially stressful restraint. However, the tethering with maternal and fetal catheterization preparation gives researchers access to the physiology of both the mother and the fetus, and even to fetal behavior. The technology and procedures, developed mainly in sheep and further adapted for baboons and rhesus
monkeys, are not new (McNamee et al., 1984; Nathanielsz et al., 1984; Stark et al., 1989). A researcher can deliver drugs and draw blood or amniotic fluid, while monitoring intrauterine pressure, blood pressures, and heart rates without having to restrain or anesthetize either the mother or the fetus. Assessing fetal behavior is even possible with electrodes implanted to measure muscle function. While not exactly replicating the human data that can be produced, the strength of the data obtained lies in the fact that the animals are freely behaving. This is not possible for human ultrasound measurements, during which the mother is usually required to lie still in a recumbent position for the duration of data collection. It is unclear whether data obtained during this artificial relaxation generalizes to maternal–fetal data during other time periods in a typical day. Therefore, each of these models has unique strengths.

Although the effects on the fetus have been assessed, postnatal development of the infants is rarely of interest. In contrast to the tethering research discussed above (Novak 2002, 2004a), the majority of tethering and catheterization studies focus on prenatal physiological questions, such as fetal cardiorespiratory measurements (Novy et al., 1971), uterine blood flow in response to intravenous cocaine administration (Morgan et al., 1991), fetal and maternal endocrine responses to reduced uteroplacental blood flow (Shepherd et al., 1992), fetal and maternal origin of leukocytes in the amniotic fluid (Macias et al., 2001), the assembly of fetal fatty acids (Su et al., 2001), and the timing, inhibition, and induction of myometrial activity and parturition (Baguma-Nibasheka et al., 2000; Farber et al., 1997; Giussani et al., 1996; Morgan et al., 1994; Sadowsky et al., 2000). To address fetal-infant continuities such as those proposed in this chapter, Novak (2002, 2004a) demonstrated the efficacy of these procedures in producing healthy, naturally delivered, full-term infants.

Using the appropriate methodologies, the role fetal experience and classical conditioning play in establishing the pacer range can be described. Hormonal responses of the mother, mediated by the placenta, represent a potentially powerful unconditioned stimulus to the fetus, provoking unconditioned hormonal, immunological, physiological, and behavioral responses in the fetus. This type of maternal–fetal communication is, initially, rudimentary and functions from the moment fetal tissues develop receptors for the maternally mediated messages. Maturation of fetal sensory systems, however, brings about new capabilities. The fetus becomes able to detect and then differentiate environmental stimuli. Under normal developmental conditions, many of these stimuli predictably precede the hormonal influences from the mother, becoming conditioned stimuli. These conditioned stimuli can include changes in the sound of the heart rate, gustatory sounds, external environmental noise, changes in heat, and somatosensory information. These stimuli can precede, and perhaps overlap, the hormonal and/or immunological disturbance that is generated in the mother and transferred to the fetus via the
placenta. Classical conditioning requires that over time and repeated exposure a link will be formed between the predictors of unconditioned stimuli, as long as the information improves the prediction of the disturbance to the fetus delivered via the placenta and umbilical cord. Repeated pairings with the conditioned stimuli will produce the response in the fetus that previously was produced only by the placentally mediated disturbance, that is, a conditioned response.

The better learned the predictor becomes, the better a poorly myelinated fetus can marshal its own resources to respond to an anticipated disturbance. The more diverse types of classically conditioned links that can be developed, the more diverse environments an organism can respond to. Somewhat paradoxically, however, the diversity of prenatal experience must not supersede the predictability of prenatal experiences, else it will interfere with the predictability necessary to establish classically conditioned links. Therefore, predictable fetal experiences may establish fetal anticipation or sensitivity to changes in its external environment. The generalization of fetal sensitivity through repetition enables a fetus to withstand a greater degree of physiological disturbance in response to environmental stimulation. The primary implication of this proposal is that prenatal psychosocial stress can be good, and is even necessary for typical development. Prenatal psychosocial stress establishes the pacer range of the fetal organism that is essential to the performance of adaptive behavior after birth.

Maternal–fetal pairs that learn this type of anticipatory sensitivity to each other will have an advantage postnatally in developing maternal–infant reciprocity and laying the groundwork for desired attachment patterns. Necessary to this hypothesis is that there are individual differences in patterns of fetal learning. This has been demonstrated for habituation learning (van Heteren et al., 2000). The development of individual differences in responsivity to environmental events has not been assessed, but is one of the primary goals of the author’s work with maternal and fetal catheterization (Novak 2002, 2004a).

The necessary formation of maternal–fetal links may possibly explain the diversity of results from the prenatal stress literature. Some stress is necessary and helps to optimize development, some has no effect, and some is detrimental. Traditionally, intensity and chronicity of stress have been argued to be the destructive components of fetal experience that cause poor developmental outcomes. The model proposed here, however, makes different predictions. Predictable forms of stress that help to classically condition mother–infant reciprocal responsiveness will not be harmful to the fetus, even in intense and/or chronically stressful situations. Only unpredictable forms of stress, in which the classical conditioning of the acute aspects of the stress is prevented, would be damaging to the fetus, perhaps even in relatively acute and modest exposures.
The proposal is not to ignore the intensity and chronicity components of stress. However, there may be other components of the stress experience, namely predictability, that need further study. For intensity and chronicity, negative outcomes in the fetus may be induced only for stimuli outside the fetal pacer range. However, within the pacer range, the predictability of fetal environmental stimuli may initiate individual differences in the pacer range itself. Furthermore, in the muted sensory world of the fetus, the rudimentary pacer range of the fetus is augmented by the dampening capacity of the womb. This reduces the significance of intensity of some stress, and emphasizes the significance of changes in maternal behavior and physiology, which may be less muted than the environment external to the mother. Further, chronic stress may not have the same effect as the frequency of stress. Frequent stressful experiences may actually assist the fetus in forming classically conditioned sensitivity to the mother. Once a chronic stress is established in the mother, fewer stimulus changes in the fetal environment may occur that require the fetus to respond. Short of physiological exhaustion in the fetus, the long-term effects may be minimal.

A caveat to the chronic stress idea is anxiety. Anxiety is often interpreted as a chronic stress state; however, this may not be the case. Instead, current conceptualizations of anxiety involve how “tightly wrapped” an individual is (DiPietro, personal communication 2003). Thus anxious mothers aren’t necessarily chronically stressed but rather in an elevated state of vigilance wherein a stress response to even low-level stimulation is highly probable. Using this rationale, maternal anxiety may be a particularly relevant form of prenatal experience because it increases the frequency and diversity of environmental events to which the mother responds. If the environmental events are predictable in nature due to the increased frequency of responding, then prenatal development is facilitated. If there is too much diversity in the type of environmental stimuli that induce maternal stress and the style of maternal responding, then negative outcomes may develop as the induction of the fetal pacer range is interfered with. Consistent with this hypothesis, DiPietro et al. (2004) report improved performance on the mental development index of the Bayley test battery in 24-month-old infants whose mothers were highly anxious during mid- to late-gestation. All the mothers were basically middle-class women with normal pregnancies. These results remained significant after controlling for depression, and stress simultaneously, and anxiousness, depression, and stress both later in pregnancy and during the prenatal period. Unfortunately, predictability was not assessed.

For the infant to attach appropriately to its mother, classical conditioning in the fetus must occur. The infant must come to be sensitive to the environment. In most cases this immediate environment is the mother. Prenatally, the link to mother as the immediate environment is even more compelling. The effect of this learning is increased sensitivity to the subtle cues in
behavior and physiology by both the mother and the fetus. Consistent with the literature that finds null and/or positive effects of prenatal stress, some disturbance of the maternal–fetal unit is necessary for the fetus to learn maternal physiological and behavioral predictors of stress. Without predictable stress during pregnancy, fetal sensitivity to the immediate environment for both reactivity and soothing purposes does not develop appropriately. Acute aspects of maternal stress responses become classically conditioned to produce anticipatory reactivity in “normally developing” fetuses. Likewise, acute aspects of the maternal soothing response (e.g., slowing of the heart rate) may also play a role. Furthermore, disturbances created by the fetus may classically condition the mother to be sensitive to her offspring.

VII. Conclusion

Pattern recognition by the fetus via simple classical conditioning may help establish a rudimentary pacer range during mid- and late-gestation. Once established, developmental complexity dissonance theory applied to both pre- and postnatal development may provide an explanation of the necessary and sufficient environmental stimuli that produce individual differences in adaptive behavior in developing organisms. As the prospects for manipulating the induction, facilitation and maintenance of individual processes via novel technologies such as stem cells, gene therapies, and reproductive technologies become more practical, both the intended and unintended effects on the development desperately need to be understood. Lessons from behavioral genetics research should be a boon to the experimental sensitivity required to assess the dynamic interactions between genes and the environment. The rodent literature has shown that environmental effects are difficult to predict, particularly with moderate effect sizes (Wahlsten et al., 2003). In nonhuman primates, that more closely approximate human cognitive, emotional, and social complexity, this difficulty is amplified because of the subtlety with which these core processes can function in a meaningful way. Regardless of species, the goal cannot be to categorize nature and nurture components of development. The dualism of this approach is invalid (Wahlsten and Gottlieb, 1997). Subjective experiences of the individual, discussed above, are one example of why approaches to understanding gene–environment interactions must be dynamic both within and between subjects. In addition, we must attempt to understand the beginnings of these dynamic interactions fetally as the nervous system becomes capable of pattern recognition. Otherwise, understanding the progressive canalization of development in response to environmental change is similar to attempting to understand an interesting novel by starting in the middle chapters of the book. The determining inductive factors in subjective experiences of an organism must be taken
into account. Whether or not these experiences can be systematically quantified is one of the many challenges addressed in this chapter. This heuristic framework provides several testable hypotheses about how organismic gene–environment interactions during pre- and postnatal development produce adaptive behavior.

Comprehensive pursuit of these ideas requires two complementary research directions: the elusive search for fetal–maternal universals, or main effects, and sensitivity to the idiosyncratic nature of truly organismic interactions, and chaotic systems. First, more experimental testing of specific gene–environment interactions is necessary to describe the complexity of developing systems. For developmentalists interested in human organismic behavior, the nonhuman primate model offers the best approximation to human development, reducing the likelihood of faulty inference (i.e., thalidomide; Hendrickx, 1973; Hendrickx and Helm, 1980; Hendrickx and Sawyer, 1978). Studies with sufficient sample sizes need to be performed to describe subtle biobehavioral similarities of moderate effect size across pregnancies and subjects. More importantly, these studies need replication, else the reliability of many findings (those significant at $p = 0.05$) is no better than the single flip of a coin.

Second, more intensive small sample size research needs to investigate the sources of individual differences. These studies will involve fewer subjects but greater resolution of any experimental effect by increasing the number of dependent variables within a response system such as measuring blood pressure, blood oxygenation, heart rate, and heart rate variability, rather than just one of these variables. In addition, these studies will achieve greater resolution by assessing more different kinds of organismic responses, such as cardiovascular reactivity, behavioral reactivity, endocrine reactivity, and metabolic reactivity, rather than just one of these dimensions.

The research suggested here opens the possibility that individual dependent variables of an organismic response such as cortisol by itself, or behavior by itself, will not ultimately prove fruitful. Each organism to be studied must be presented with the same situation multiple times, and with multiple situations. On some dependent variables unique results will occur each time, whereas others will exhibit trends. The patterns exhibited across time and individuals are higher order dependent variables. Individuals with different patterns across presentations of the same environment may prove just as informative for the advancement of theory as common patterns within and across individuals. Consistent with this idea, Corneal and Nesselroade (1994) have used factor analyses of multiple personality assessments in humans to demonstrate elusive consistency in personality variables over time and across situations. In their analyses, the number of different factors on which the assessments loaded for a given individual became the higher order dependent variable and transcended many of the subjectivity concerns.
inherent in personality theory. Shoda (1999) and Shoda and Mischel (2000) proposed that patterns demonstrated by individuals across multiple dependent variables and situations reveal a “behavioral signature” for an individual that is not evident in a particular effect on a particular variable at a given time. For developmental biobehavioral research, these systems or organismic methodologies will allow analyses to further grapple with situational influences and the potential confounds of how organisms come to perceive their environment.

References


2. Fetal Experience


2. Fetal Experience


Paradoxical Role of Methyl-CpG-Binding Protein 2 in Rett Syndrome

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Acknowledgments
References

I. Introduction to Rett Syndrome

A. Clinical Features

Rett syndrome (RTT) is a neurodevelopmental disorder representing one of the most frequent causes of severe mental retardation in females (Armstrong, 1997; Ellaway and Christodoulou, 1999). A striking feature of RTT is that infant girls develop normally until 6 to 18 months of age but then develop a progressive loss of neurodevelopmental milestones. Clinical features include deceleration of head growth, loss of purposeful hand
movements, ataxia, loss of vocalization skills, autistic features, seizures, and respiratory dysfunction. A preserved speech variant (PSV) form of RTT is also observed in which some girls recover the ability to speak in short phrases and regain some hand use (Zappella et al., 2001). As the clinical features overlap with other neurodevelopmental disorders, the necessary criteria established for diagnosis with RTT include the following: apparently normal prenatal and perinatal development until 6 months; normal head circumference at birth, followed by deceleration from 5 months to 4 years of age; loss of acquired purposeful hand movements from 6 to 30 months, associated with communication dysfunction and social withdrawal; development of severely impaired language and psychomotor skills; stereotypic hand movements of “hand wringing”; and gait and truncal apraxia at 1 to 4 years of age (Armstrong, 1997). The frequency of RTT is estimated to be between 1 in 10,000 and 1 in 15,000 (Van den Veyver and Zoghbi, 2000).

B. Genetic Inheritance

RTT was predicted to be an X-linked dominant disorder on the basis of its strong predominance in females. The genetic defect in RTT was mapped to Xq28 (Sirianni et al., 1998) and mutations were identified in the gene MECP2 (Amir et al., 1999). MECP2 encodes the methylated CpG-binding protein 2 (MeCP2) (Lewis et al., 1992; Meehan et al., 1992). Subsequent studies have identified MECP2 mutations in approximately 80% of sporadic classic RTT patients (Amir et al., 2000; Bienvenu et al., 2000; Buyse et al., 2000; Cheadle et al., 2000; Huppke et al., 2000; Xiang et al., 2000), and 55% of PSV patients (Zappella et al., 2001).

Interestingly, several reports have demonstrated MECP2 mutations in neurodevelopmental disorders other than RTT, including Angelman syndrome, X-linked mental retardation, and severe neonatal encephalopathy (Clayton-Smith et al., 2000; Couvert et al., 2001; Orrico et al., 2000; Villard et al., 2000; Watson et al., 2001). These findings suggest that the scope of diseases associated with MECP2 mutations may extend beyond RTT to other forms of X-linked mental retardation and severe neurodevelopmental disorders. Although few cases of autism with MECP2 mutations have been described (Beyer et al., 2002; Carney et al., 2003; Lam et al., 2000), several large studies have determined that mutations or polymorphisms in the coding region of MECP2 occur at a low frequency in autistic patients (Beyer et al., 2002; Lobo-Menendez et al., 2003; Vourc’h et al., 2001).

The first chimeric mice made with MeCP2-deficient embryonic stem cells died in utero, suggesting an essential role for the gene in global prenatal development (Tate et al., 1996). Two groups then used Cre–lox recombination to create MeCP2-null mice demonstrating a Rett-like phenotype (Chen et al.,...
1. Paradoxical Role of MeCP2 in Rett Syndrome

Both male hemizygous and female heterozygous mice are viable and show no apparent phenotype at birth, but males begin to develop neurological symptoms at about 4–6 weeks of age and usually die within 10 weeks. Female MeCP2+/- mice do not develop a comparable phenotype until several months of age. Interestingly, a targeted deletion of MeCP2 in postnatal central nervous system (CNS) neurons led to a similar phenotype. Brains from mutant mice showed a significant reduction in weight and neuronal and nuclear size (Chen et al., 2001). A report of a mouse model with MeCP2 truncation mutations that mimic those found in RTT further demonstrates that these mice experience a phenotypic delay until 5 weeks of age, showing several features of RTT including motor impairments, seizures, autistic behaviors, and hypoactivity (Shahbazian et al., 2002a). These results have demonstrated that MeCP2 is required for the normal functioning of the mature mammalian nervous system and MeCP2 mutation is sufficient to cause the RTT-like phenotype in mice.

II. CpG Methylation and the Role of MeCP2 in Chromatin Organization

CpG is the least frequently represented dinucleotide in the mammalian genome because of deamination reactions that convert methylated cytosines to uracils (Bestor and Tycko, 1996). Unmethylated CpG sites are conserved in “CpG islands” that tend to be clustered at the 5’ end of many mammalian genes. Although the focus of much DNA methylation and chromatin research has been on CpG islands that become methylated during the regulation of specific genes, it is important to recognize that most DNA methylation in the genome is in heterochromatic and repetitive sequences that are not in immediate proximity to actively transcribed genes.

Methylation of CpG sites at the 5’ end of a gene is usually associated with transcriptional silencing (Bestor and Tycko, 1996). Because of leaky basal transcription of genes in actively transcribed genomic regions, a simple lack of transcription factors is not enough to completely silence a gene. The identification of MeCP2 as a methyl-CpG-binding protein was a critical bridge to understanding the link between methylation at the DNA level and functional changes in proteins involved in chromatin organization that orchestrate epigenetic transcriptional silencing.

MeCP2 was originally isolated from rat brain nuclear extracts in a screen to identify novel proteins that selectively bound probes containing methylated CpGs (Lewis et al., 1992). MeCP2 localization to transcriptionally inactive heterochromatic regions of the nucleus depended on DNA methylation and the presence of a 85-amino acid methyl-CpG-binding domain (MBD) toward the N-terminal end of the protein (Nan et al.,
A larger family of methyl-CpG-binding proteins was isolated on the basis of homologous MBD domains and include MBD1, MBD2, MBD3, and MBD4 (Hendrich et al., 1999). Another region, termed the transcriptional repression domain (TRD), was identified in MeCP2 and found to be capable of long-range repression (Nan et al., 1997). RTT mutations in MECP2 are found throughout the gene, but tend to fall into three major categories: missense mutations in the MBD, nonsense mutations that truncate the protein before or around the TRD, and deletions or complex rearrangements in the C terminus of the protein with unknown function (reviewed in Amir et al., 2000).

Modifications such as acetylation, methylation, and phosphorylation of specific sites on core histone H3 and H4 constitute important differences between active and inactive chromatin (Jenuwein and Allis, 2001). Actively transcribed genes are frequently associated with acetylation of K14 of H3 and K5 of H4, whereas inactive regions are deacetylated at these residues and methylated on K9 of histone H3. MeCP2 interacts with the transcriptional repressor Sin3A through the TRD and histone deacetylase (HDAC), providing a mechanism for the transcriptional repression of genes with methylated CpG sites (Jones et al., 1998). MeCP2 has been shown to repress transcription of methylated promoters in vitro and of simian virus 40 (SV40) or GAL4-containing promoters in vivo (Nan et al., 1997; Yu et al., 2000). In addition to associating with histone deacetylase activity, MeCP2 has been shown to associate with histone methyltransferase activity (Fuks et al., 2003). MeCP2 may therefore direct histone H3 K9 methylation to methylated DNA, potentially repressing transcriptional activity. Another study showed association of MeCP2 with the maintenance DNA methyltransferase Dnmt1 in the absence of HDAC1 and Sin3A when bound to hemimethylated DNA, providing a mechanism by which MeCP2 may target newly replicated DNA for maintaining methylation and transcriptional inactivity (Kimura and Shiota, 2003).

Several animal models have established that proper CpG methylation appears to be essential for neuronal development and survival in the CNS (Tucker, 2001). Interestingly, the brain has among the highest levels of both methylated cytosine (Ehrlich et al., 1982) and Dnmt1 expression (Inano et al., 2000). Loss of Dnmt1 in neuronal precursors results in highly demethylated neurons and glia that are rapidly eliminated (Fan et al., 2001). Interestingly, Dnmt1 mutations targeted to postmitotic CNS neurons have no apparent phenotype, in contrast to MeCP2 mutations (Chen et al., 2001; Guy et al., 2001), and despite high levels of Dnmt1 in normal brain (Inano et al., 2000).

Genes involved in DNA methylation and chromatin organization are also included on a growing list of human disease genes that cause mental retardation (Hendrich and Bickmore, 2001). As brain development involves
environmental as well as genetic factors, it should not be surprising that epigenetic pathways are implicated in the etiology of many neurodevelopmental disorders. Further understanding of the role of MeCP2 in the pathophysiology of RTT has therefore enormous promise in serving as a model by which insights into other more genetically complex neurodevelopmental disorders including mental retardation, epilepsy, and autism may be found. In spite of the seemingly well-characterized models for MeCP2 function and the identification of MECP2 mutations in RTT, the field has had several unexpected twists and turns in the rapidly unraveling story of how mutations in MECP2 cause RTT.

III. The Paradoxes

A. Why Does Rett Syndrome Affect Primarily Females?

Originally, RTT was thought to be an X-linked dominant inheritance with male lethality because all clinical cases of RTT involved females (Van den Veyver and Zoghbi, 2000). The first description of an Mecp2-null chimeric mouse also showed embryonic lethality, supporting the original hypothesis of male embryonic lethality (Tate et al., 1996). In rare familial cases of RTT, however, MECP2 mutations do not exclusively determine phenotype, as mothers of RTT siblings also were heterozygous for the same MECP2 mutation but did not exhibit the phenotype due to skewed X inactivation (Wan et al., 1999). Furthermore, boys born to these families who inherited the same MECP2 mutation as their sisters with RTT showed severe neonatal encephalopathy and death by 1 year of age (Schanen and Francke, 1998; Schanen et al., 1997; Villard et al., 2000; Wan, 1999). Rare cases of boys with RTT and somatic mosaicism for an MECP2 mutation (Clayton-Smith et al., 2000) or Klinefelter’s syndrome (47, XXY) (Hoffbuhr, 2001; Schwartzman et al., 1998) have also been described, demonstrating that heterozygosity of the MECP2 mutation is a defining feature of classic RTT. Therefore, although the same MECP2 mutation certainly causes a more severe phenotype in boys compared with girls, inheritance of MECP2 mutations does not necessitate embryonic lethality in the hemizygous male or an RTT phenotype in the heterozygous female.

1. Role of X Chromosome Inactivation in the Development of Rett Syndrome

MECP2 is subject to X chromosome inactivation (XCI), so each cell within a female’s body expresses only one of the two possible X chromosome alleles. Because XCI is usually a random process, normal females are expected to
have approximately 50% of their cells expressing genes from the maternal X chromosome and 50% from the paternal X. If there is a negative growth phenotype associated with an allele on one X chromosome in the early embryo when random XCI is established, however, nonrandom or skewed XCI may occur (>80 or <20% of cells with an inactive allele), usually selecting for the wild-type allele as the active X chromosome. Females with a seemingly normal phenotype may have skewed XCI that is not realized until they have increased rate of miscarriage in early pregnancy (Robinson et al., 2001; Uehara et al., 2001) or have children born with a mutation in an X-linked gene such as MECP2. Skewed XCI can therefore explain how the mother of RTT children may have the genotype of heterozygous MECP2 mutation but not express the phenotype (Wan et al., 1999), or how identical twins can be discordant for RTT (Bruck et al., 1991; Migeon et al., 1995). Most RTT patients, on the other hand, have random XCI patterns in both blood and brain (Amir et al., 2000; Shahbazian et al., 2002c; Zoghbi et al., 1990).

2. Male Lethality versus Paternal Inheritance

Although the familial cases of RTT have been useful for determining the role of XCI in the phenotype of RTT, the vast majority (>99%) of RTT cases have sporadic mutations in MECP2 that are not found in either parent (Zoghbi et al., 1990). Although maternal transmission and nonrandom maternal XCI were observed in most familial cases, the possibility remained that RTT was sex limited due to preferential inheritance of paternal mutations in sporadic cases of RTT (Thomas, 1996). An embryo that inherits an X chromosome from its father will become a female, whereas a maternally contributed X chromosome could result in either male or female. The majority of mutations in MECP2 are due to C-to-T conversions in CpG dinucleotides (Dragich et al., 2000). DNA in sperm is much more highly methylated than the same sequences in oocytes because of the need for greater compaction. In addition, methylated cytosines become converted to uracil, which is not as easily recognized by DNA repair processes as the direct conversion from C to T that occurs on unmethylated cytosines. Therefore, sperm DNA is predicted to be more susceptible to mutations in a highly CG-rich gene such as MECP2 (Thomas, 1996). Two studies directly addressed the hypothesis that the reason RTT occurs more frequently in females is because mutations occur more frequently in the male genome (Girard et al., 2001; Trappe et al., 2001). One study found paternal inheritance of MECP2 mutations in 27 of 28 patients (Trappe et al., 2001), whereas the other found such mutations in 5 of 7 patients (Girard et al., 2001), for a total of 91% of the RTT mutations derived from the paternal allele. These results confirmed the hypothesis that the higher occurrence of RTT in females may be in part due to increased paternal inheritance of MECP2 mutations.
3. **MECP2 Mutations in X-Linked Mental Retardation**

Another interesting possibility is that perhaps the use of the strict clinical criteria for diagnosing RTT (described in Section I.A) selects for a phenotype found primarily in female heterozygotes of severe MECP2 mutations with random XCI and excludes other phenotypes also due to MECP2 mutations. Several studies screening for MECP2 mutations in familial cases of male X-linked mental retardation (XMR) with no known genetic defects found mutations in MECP2 in ~2% of the patients (Couvert et al., 2001; Dotti et al., 2002; Orrico et al., 2000). Interestingly, the mutations found in XMR were missense rather than nonsense, and different from those found in RTT, suggesting a milder phenotype associated with these mutations than that observed with the classic RTT MECP2 mutations. The most common mutation in XMR is an A140V mutation not previously observed in female RTT cases, but linked to mild mental retardation in females within the same pedigree as more severely affected males (Orrico et al., 2000; Dotti et al., 2002). The MECP2 A140V mutation was also found to cause PPM-X syndrome, characterized by mental retardation, manic–depressive psychosis, parkinsonian features, and macroorchidism, and suggesting MECP2 A140V as a “hot spot” for mutations in XMR (Klauck et al., 2002).

Genotype–phenotype correlations have been difficult to assess in RTT because of the influence of XCI ratios in heterozygous females (Shahbazian and Zoghbi, 2002). In males, however, the genotype–phenotype correlation may be more straightforward: mild mutations such as A140V result in a normal life span with moderate to severe mental retardation, RTT mutations such as R168X result in severe neonatal encephalopathy and early death, and presumably very early truncation mutations would result in embryonic lethality, as these have not been observed in males or females (Dragich et al., 2000; Shahbazian and Zoghbi, 2002). These findings suggest that a wider screen for MECP2 mutations in mental retardation may uncover additional mutations in males or perhaps heterozygous females with milder mutations or favorable XCI ratios.

B. Why Is the Phenotype of Rett Syndrome Restricted to the Central Nervous System?

1. **Northern Blots Demonstrate Ubiquitous Expression**

*MECP2* is observed to be ubiquitously expressed in different tissues by Northern blot analysis (Coy et al., 1999; D’Esposito et al., 1996; Reichwald et al., 2000; Shahbazian et al., 2002b). Some differences in expression are observed between different tissues, but this is complicated by the presence of at
least two different transcripts. A 1.9-kb band represents the predominant short form of MECP2 that is more highly expressed in heart, kidney, and skeletal muscle. An extremely large (10-kb) band is also present and reflects an alternatively polyadenylated transcript that includes the 1.5-kb coding region plus an 8.5-kb untranslated region (3' UTR) (Coy et al., 1999; Reichwald et al., 2000). Brain exhibits more of the long MECP2 transcript than do other tissues, but it is also ubiquitously expressed. No difference in stability was observed between the two MECP2 transcripts (Reichwald et al., 2000). In addition, no correlation between the level of expression of either transcript by Northern blot, and of MeCP2 protein expression by Western blot, was observed, making the tissue-specific differences in MECP2 transcript levels difficult to resolve (Shahbazian et al., 2002b). Therefore, MeCP2 protein expression is not directly linked to level of transcript and may be regulated by more complex posttranscriptional mechanisms.

2. Immunofluorescence Demonstrates Heterogeneity in MeCP2 Protein Expression

Another issue that complicates the analysis of MECP2/MeCP2 expression by Northern or Western blots is the issue of cellular heterogeneity. Heterogeneity at the level of MeCP2 expression has been observed by immunofluorescence and immunohistochemistry in human, monkey, rat, and mouse adult brain tissues (Akbarian et al., 2001; Jung et al., 2003; LaSalle et al., 2001; Shahbazian et al., 2002b). An example is shown in Fig. 1A of adult mouse cerebral cortex stained for MeCP2 (red), with a nuclear counterstain (green). MeCP2 is localized to the nuclear heterochromatin in all cells, but at least two distinct populations are observed. Neuronal nuclei expressing high levels of MeCP2 (red arrows) have been termed “MeCP2^{hi},” whereas the remaining cells with a low but detectable level of MeCP2 are called MeCP2^{lo} (green arrows) (LaSalle et al., 2001). In other studies, MeCP2 expression is often not detected in the MeCP2^{lo} population because of decreased sensitivity of experimental methods, so these cells are often described as “negative” for MeCP2 expression (Akbarian et al., 2001; Jung et al., 2003; Shahbazian et al., 2002b).

The new technology of laser scanning cytometry has allowed us to quantitate MeCP2 expression in individual cells of tissue sections and to localize subpopulations of cells back to specific tissues or regions (LaSalle et al., 2001). While virtually all cells in normal brain are positive for MeCP2 compared with appropriate controls, in the adult cerebral cortex neurons are divided roughly equally between MeCP2^{lo} and MeCP2^{hi} populations. Regional variations in MeCP2 subpopulations are observed, with MeCP2^{hi} cells most frequent in layer IV of the cerebral cortex and MeCP2^{lo} cells most frequent in the granular layer of the cerebellum. Although glial
cells in the cerebral cortex are all MeCP2lo, white matter glial cells are also heterogeneous for MeCP2 expression. Other studies have also found an absence of elevated MeCP2 expression in glial cells of the cerebral cortex, demonstrating that neurons make up the predominant subpopulation of MeCP2hi cells in the CNS (Jung et al., 2003; Shahbazian et al., 2002b).

3. Elevated MeCP2 Expression in Subpopulations of Cells in the CNS

To address the question of whether this subpopulation of high MeCP2-expressing cells was limited to the CNS or was observed in other tissues showing MeCP2 expression by Western blot, the quantitative laser scanning cytometry (LSC) approach was also useful. Multiple tissue arrays containing different human or mouse tissues were analyzed for mean MeCP2 expression and the percentage of MeCP2hi cells (LaSalle et al., 2001). For both species, the mean MeCP2 expression and percentage of MeCP2hi cells were
significantly higher in CNS compared with non-CNS tissues. This CNS-specific MeCP2\textsuperscript{hi} expression can also be observed in a whole mouse pup LSC image, shown in Fig. 1B. The scattergram represents the x and y positions of each contoured cell analyzed by LSC and mapped back to the original tissue location. MeCP2\textsuperscript{hi} cells are colored red, MeCP2\textsuperscript{lo} cells green, and negative cells blue. In a 3-day-old pup, the MeCP2\textsuperscript{hi} cells localize to the brainstem and spinal cord, which are developmentally the first to show the MeCP2\textsuperscript{hi} phenotype (Shahbazian and Zoghbi, 2001).

These results demonstrate that MeCP2 is highly expressed in a subpopulation of cells within the CNS and begin to explain how the phenotype of RTT and the \textit{Mecp2}\textsuperscript{-null} mouse can be CNS specific in spite of ubiquitous MECP2/\textit{Mecp2} expression. These findings also suggested that perhaps \textit{MECP2} mutations were manifested only in MeCP2\textsuperscript{hi} neurons. In other words, MeCP2 may be dispensable in most tissues with a low level of expression, but the CNS neurons may require greater amounts of MeCP2 and therefore be more affected by loss-of-function mutations.

C. Why Is the Phenotype of Rett Syndrome Restricted to Postnatal Development?

1. Northern Blots Demonstrate Fetal and Adult Expression

In line with this hypothesis, one would also expect that the reason for the delay in the development of clinical features in RTT and a detectable phenotype in \textit{MeCP2}-null mice would be because \textit{MECP2/Mecp2} is developmentally expressed at a higher level in the adult. Northern blot data again did not support this hypothesis, as \textit{MECP2} was highly expressed in both fetal and adult tissues (Coy et al., 1999). The long (10-kb) transcript was more highly expressed in fetal compared with adult tissues of all types, but otherwise there was equivalently high transcriptional expression in both developmental stages. \textit{In situ} hybridization showed high levels of expression in the neonatal brain from both mouse and rat (Coy et al., 1999; Jung et al., 2003).

2. Increase in MeCP2\textsuperscript{hi} Cells throughout Postnatal Brain Development

Immunohistochemical studies in mice and humans demonstrated a spatial and temporal distribution of MeCP2 expression and the emergence of the MeCP2\textsuperscript{hi} [or immunohistochemistry (IHC)-detectable expression] population (Shahbazian et al., 2002b). We also performed LSC analysis on a developmental tissue array containing human brain and kidney sections from fetal, infant, juvenile, and adult samples (Balmer et al., 2003). A significant
correlation between age and MeCP2 level, population heterogeneity, and the percentage of MeCP2 \( \text{hi} \) cells was observed in the cerebral cortex. Interestingly, although both kidney and brain showed the same low-level expression of MeCP2 in fetal tissues, the gradual increase in MeCP2 expression and the MeCP2 \( \text{hi} \) population with age was specific to the postnatal CNS. An inverse correlation was observed in the usage of the long \( MECP2 \) transcript with age, suggesting that alternate polyadenylation may partially regulate the developmental changes in MeCP2 protein expression (Balmer et al., 2003). In agreement with Northern blot analyses, however, the switch from predominant usage of the long to short \( MECP2 \) transcript was also observed in non-CNS tissues that do not show an increase in MeCP2 protein expression, suggesting that developmental increases in MeCP2 expression cannot be entirely due to alternate polyadenylation (Balmer et al., 2003; Coy et al., 1999). Combined analyses of MeCP2 immunofluorescence and \textit{in situ} hybridization of both \( MECP2 \) transcripts suggest that multiple transcriptional and posttranscriptional pathways regulate the complex developmental expression patterns of MeCP2 in the human postnatal brain (Samaco et al., unpublished data).

3. Conclusions from MeCP2 Expression Studies

These combined results on \( MECP2/\text{Mecp2}/\text{MeCP2} \) expression studies have raised more questions than they have answered, but begin to shed light on the reasons for the CNS specificity and postnatal delay in the phenotype associated with MeCP2 loss-of-function. At the protein level, MeCP2 shows highly complex developmental regulation, with differences in expression in different cells, tissues, brain regions, and stages of development. The regulatory mechanisms that orchestrate the complex expression changes in MeCP2 are only beginning to be understood, with much work ahead to identify tissue-specific and developmental-specific factors that regulate transcriptional and posttranscriptional control of MeCP2. The implication for understanding RTT, however, is that elevated MeCP2 expression appears linked to postmitotic neuronal maturational differentiation. This hypothesis would fit with the original pathological studies that described defects in dendritic branching and the overall number of synapses in RTT brains (Armstrong et al., 1995, 1998). Studies on mouse olfactory neurons, whose stages of development can be experimentally investigated, also support this hypothesis, as increases in MeCP2 expression depended on synaptogenesis (Cohen et al., 2003). Therefore, although \( MECP2 \) is expressed ubiquitously, perhaps it is critical in the brain only when maturing neurons are making important connections.
D. Why Is the Timing of the Rett-Like Phenotype in the MeCP2-Null Mouse Model Developmentally Delayed Compared with Humans?

1. MeCP2 Is Involved in Neuronal Maintenance Rather than Maturation

As mentioned in the introduction (Section I.B), the MeCP2-null mouse model seems at first glance to mimic human MECP2 mutations in RTT well, as the impairments are specific to the CNS and delayed until postnatal development (Chen et al., 2001; Guy et al., 2001). The timing of the phenotype in these models, however, appeared to be similar to humans chronologically rather than developmentally, prompting the authors to propose that MeCP2 plays a role in neuronal maintenance rather than maturation. Male MeCP2-null mutant mice develop a phenotype between 3 and 6 weeks, with death by 4–12 weeks (Chen et al., 2001; Guy et al., 2001). Female heterozygous mice were more variable in expressing the phenotype, with 40% showing symptoms at 4 months and 70% by 10 months of age (Guy et al., 2001). Because this is adulthood for the mouse, a comparison with female RTT patients who develop symptoms in infancy (6 to 18 months) would suggest a chronologic rather than developmental overlap. Many other possible explanations have been proposed to explain the delayed phenotype in the MeCP2-null mouse model, and these are outlined below.

2. The Phenotype of MeCP2 Deficiency Is Less Severe or Noticeable in the Mouse than Human

As with other mouse models for neurodevelopmental disorders, the phenotype of a cognitive delay may be less readily apparent in mice than in humans, concerning whom milestones for cognitive and language development are well characterized and monitored. Perhaps female mice heterozygous for MeCP2-null mutations do exhibit earlier, subtle behavioral and cognitive defects that are not detected by the rudimentary tests performed thus far. In addition, mice may simply be able to handle MeCP2 deficiency better than do humans because of a lack of higher cognitive processes such as language development. These possibilities need to be investigated further by behavioral and cognitive neuroscientists interested in these processes, as the MeCP2-null mouse model is now readily available.

3. The Phenotype of MeCP2 Deficiency in Mouse is Different than MECP2 Dysfunction in Humans

Another argument is that perhaps the reason for the difference in age of onset between mouse and human is that MeCP2 dysfunction observed in RTT mutations is inherently different from the complete loss of expression in MeCP2-null mice. Truncated protein products of nonsense MECP2
mutations are expressed in lymphocytes and brain from RTT patients (Balmer et al., 2002; LaSalle et al., 2001) and the truncated \textit{Mecp}^{2308} mutation in mouse is expressed and localizes normally to heterochromatin (Shahbazian et al., 2002a). The less severe phenotype observed in male \textit{Mecp}^{2308/y} mice compared with male \textit{Mecp}2-null mice, however, would argue against the partial function of MeCP2 retained in RTT mutations being more detrimental than a complete loss of the protein observed in the \textit{Mecp}2-null mouse model (Chen et al., 2001; Guy et al., 2001). Additional mutant mouse models engineered with other RTT mutations would be useful in determining further genotype–phenotype correlations in the mouse.

4. The Developmental Pattern of MeCP2 Expression Is Different in Mice than in Humans

The argument made in the previous section was that the reason symptoms are delayed in RTT and \textit{Mecp}2-null mice is that MeCP2 shows complex developmental regulation and mature neurons have the highest expression of MeCP2 and are therefore most affected by mutation. Perhaps the developmental expression of MeCP2 in the important brain regions of the mouse is delayed relative to the human. Our data on quantitative localization of MeCP2 on whole mouse sagittal sections at different developmental stages would support this hypothesis (Braunschweig et al., unpublished data). Emergence of the phenotype in \textit{Mecp}2-null males occurs when the MeCP2\textsubscript{hi} population begins to emerge in the cerebral cortex, olfactory bulb, and cerebellum. Continued increases are observed in the MeCP2\textsubscript{hi} population throughout the mouse brain from 7 through 40 weeks of age, suggesting that elevated MeCP2 expression associated with neuronal maturation continues well into adulthood in the mouse.

5. Female Mice Exhibit Nonrandom XCI

On the basis of what is currently known about the importance for females of the relevance of XCI ratios to the exhibition of the classic symptoms of RTT (Section III.A), another reason why female \textit{Mecp}2-null mice do not exhibit symptoms developmentally as early as do RTT girls could be because they have nonrandom XCI. Variable nonrandom XCI may also help explain the heterogeneity between female heterozygous mice that differ in their display of RTT-like features. This argument would also suggest that \textit{Mecp}2 mutations within cells of the early embryo would exhibit a subtle phenotype enough to be selected against during early developmental cell divisions. Therefore, unlike female RTT patients who exhibit random XCI, mosaic female mice with fewer \textit{Mecp}2-mutant-expressing neurons may exhibit a milder and developmentally delayed expression of the phenotype.
E. Why Is There No Evidence of the Disrupted Repression of Methylated Genes in RTT Tissue Samples or the Mecp2-Null Mouse?

This is perhaps the most perplexing paradox for investigators interested in the biochemical function of MeCP2 as well as for those interested in the molecular pathogenesis of RTT. Because MeCP2 had been well characterized as a transcriptional repressor, the prediction was that mutation of MECP2/Mecp2 in RTT tissues and Mecp2-null mice would result in a long list of methylated genes the transcription of which was increased by MeCP2 loss-of-function. Experiments using transfected methylated constructs, including imprinted genes and proviral sequences, have clearly demonstrated MeCP2 as a transcriptional repressor (Kaludov and Wolffe, 2000; Lorincz et al., 2001; Muller et al., 2000; Nan et al., 1997; Yu et al., 2000). At this juncture, however, no endogenous methylated genes have been described that require MeCP2 for silencing. The real question that therefore remains is whether transcriptional repression is a required function of MeCP2 or whether MECP2 mutations in RTT may reveal additional previously unknown functions for this perplexing molecule.

1. Genome-Wide Analyses

Gene expression profiling experiments appear at first glance to be the most straightforward way to investigate transcriptional differences between MECP2/Mecp2 mutant and wild-type cells and tissues. Gene expression profiling can be challenging in the CNS (Luo and Geschwind, 2001), however, especially in light of the complexity of MeCP2 expression in different tissues and stages of development (outlined in Sections III.B and III.C). A report on gene expression profiling between RTT and control brain demonstrated that less than 5% of total genes were dysregulated in RTT brain and found a greater frequency and consistency of downregulated than upregulated genes (Colantuoni et al., 2001). Glial transcripts were upregulated while neuron-specific transcripts were downregulated, further suggesting a role for MeCP2 in neuronal maturation rather than global transcriptional repression.

The Mecp2-null mouse model was thought to be a preferred system for gene expression profiling as variables such as genetic background, tissue sampling and storage, and age can be controlled. Transcriptional profiling experiments on oligonucleotide microarrays also failed to show evidence of Mecp2 deficiency leading to global transcriptional repression, but did find subtle changes in gene expression by more sensitive statistical analyses (Tudor et al., 2002). None of the dysregulated genes appeared to be regulated by methylation, however, leading the authors to conclude that they may be downstream targets of methylated genes directly targeted by MeCP2.
Because the cellular and transcriptional complexity of mammalian brain tissue is expected to complicate the interpretation of gene expression profiling, perhaps a more straightforward approach is to investigate gene expression differences associated with MeCP2 dysfunction in a cell culture system. Clonal cell lines from RTT fibroblast and lymphoblast cultures have been derived so that MECP2-mutant and wild-type expressing cells from the same individual can be compared (Traynor et al., 2002). These studies also demonstrate that MeCP2 deficiency did not lead to global dysregulation of gene expression, although a few genes were found with differential expression. However, because developmentally acquired elevated MeCP2 expression is not observed in immune or epithelial tissues, perhaps these tissue types are not the most useful for determining the role of MeCP2 in regulating gene expression during neuronal maturation.

These three genome-wide transcriptional profiling experiments using different MeCP2-deficient tissues are therefore in agreement in demonstrating that the essential function of MeCP2 is not as a global transcriptional repressor. The hypothesis that MeCP2 may still regulate a small number of methylated genes remains. Different dysregulated genes were found in each of these studies, however, so their potential relevance to the pathogenesis of RTT still remains to be determined. Perhaps future transcriptional profiling experiments designed to test the effect of Mecp2 mutations during neuronal maturation may be more informative for identifying direct targets of MeCP2 rather than downstream effects.

2. Methylated and Parentally Imprinted Gene Analyses

Targeted testing of specific methylated genes for dysregulation in MECP2/Mecp2 mutant tissues or cell lines is another approach that has been taken with somewhat variable results. Parentally imprinted genes, in which one parental allele is silenced by methylation (Lalande, 1996), were obvious potential targets for MeCP2. Paternally imprinted H19 had been a candidate for regulation by MECP2 in human and mouse because of association with the differentially methylated domain and repression of transfected and methylated H19 constructs by MeCP2 (Drewell et al., 2002). Our investigation of allelic expression of three imprinted genes in lymphocyte clones (SNRPN, IPW, and IGF2) and five imprinted genes in RTT brain (SNRPN, IPW, NEC, IGF2, and H19) failed to find biallelic expression of imprinted genes associated with MECP2 mutation (Balmer et al., 2002). One study showed apparent increased expression of H19 in the Mecp2-null mouse compared with wild-type controls, but specific derepression of the normally silent paternal allele was not demonstrated, making it difficult to interpret these results as loss of methylated repression (Fuks et al., 2003). Nonimprinted genes have also been tested in MECP2-mutant cells and no evidence of
altered expression of methylated genes \textit{IFNG} or \textit{LINE-1} retrotransposons has been observed (Balmer \textit{et al.}, 2002).

These targeted approaches of testing individual methylated or imprinted genes do not rule out the possibility that methylated gene targets will be found for MeCP2, but they continue to argue against the original model of MeCP2 as a global transcriptional repressor. As imprinted genes are regulated by many different mechanisms and several show conditional imprinting in specific tissue or stages of development (Lalande, 1996), it remains possible that MeCP2 may play a role in the more long-range repression of imprinted genes, particularly those with imprinting specific to the postnatal brain.

F. \textbf{Why Are the Nuclear Changes Observed in RTT Brains and \textit{Mecp2}-Null Mice Inconsistent with MeCP2 as a Global Transcriptional Repressor?}

1. \textbf{Brain Weight and Nuclear Area Are Smaller}

The most noticeable pathology of both RTT patients and the \textit{Mecp2}-null mouse is that the brain size is smaller than that of age-matched controls (Armstrong, 1997; Chen \textit{et al.}, 2001). In the mouse \textit{Mecp2}-null model, the hippocampal CA2 neurons were significantly smaller than those of wild-type littermates and the nuclei of neurons throughout the CNS were noticeably smaller (Chen \textit{et al.}, 2001). In addition, in heterozygous female RTT brains, neurons in layers III and V of the frontal, motor, and inferior temporal cortex were smaller, with shorter apical and basilar dendritic branches (Armstrong \textit{et al.}, 1995, 1998). These pathologic observations are inconsistent with global changes in nuclear chromatin that were predicted from MeCP2 dysfunction. For instance, global increases in histone acetylation would be predicted to increase transcription throughout the nucleus, resulting in larger, more euchromatic nuclei.

The observations of smaller, more heterochromatic nuclei in the \textit{Mecp2}-null mouse and RTT brains are therefore counterintuitive. In addition, Fig. 1A shows that MeCP2$^{lo}$ neurons (green arrows) in normal adult brain are characterized by small heterochromatic nuclei whereas the MeCP2$^{hi}$ neurons (red arrows) have larger nuclear area, more euchromatin (open spaces in nuclear counterstain), and more prominent nucleoli (reflecting increased transcription of ribosomal genes). So why does increased expression of MeCP2 in mature neurons correlate with nuclear and neuronal phenotypic changes reflecting a more transcriptionally active state?
2. Histone Acetylation is Higher but Inconsistent

A few biochemical analyses of histone acetylation in the MECP2/Mecp2 mutant state have been examined, with somewhat conflicting results. A comparison of MECP2 mutant and wild-type expressing lymphoblast clones revealed higher levels of histone H4 but not H3 acetylation levels associated with MECP2 mutations (Wan et al., 2001). Analysis of nontransformed T lymphocyte clones failed to find differences in acetylated histone H4 or H3 levels between MECP2 mutant and wild-type expressing cells (Balmer et al., 2002). The Mecp2<sup>308/y</sup> mouse model allowed the investigation of histone acetylation in multiple tissues and demonstrated a significantly higher level of histone H3 acetylation in spleen, cortex, and cerebellum in mutant compared with wild-type tissues (Shahbazian et al., 2002a). Acetylated histone H4 levels, however, remained unaltered. Association of MeCP2 with changes in acetylation of histone H3, but not H4, have also been observed in vitro in investigations of methylation-mediated proviral transcriptional silencing (Lorincz et al., 2001).

In trying to interpret the histone acetylation results with the cytologic and pathologic observation of the MECP2/Mecp2 mutation state, it is important to consider the complexity of histone H3 and H4 posttranslational modifications (Jenuwein and Allis, 2001). Investigating one modification in the absence of other chromatin changes may not provide the whole picture of global chromatin and transcriptional changes within the nucleus. Although these combined results provide good evidence of MECP2/Mecp2 mutations being associated with distinct chromatin changes, the full characterization of these changes will require additional investigation at both the biochemical and cytological levels.

IV. A Reexamination of the Role of MeCP2 in the Pathogenesis of Rett Syndrome

In light of the many paradoxes that have emerged in the first 3 years since the finding of MECP2 as the genetic cause of RTT (Amir et al., 1999), it is perhaps time to develop new models that incorporate the new developments in the field. The models of MeCP2 as a regulator of chromatin organization at the molecular level need now to be incorporated with the molecular pathogenesis of RTT as a disorder of arrested development of the CNS. The promise of such interdisciplinary cross-talk is that all investigators stand to learn more about the role of this important molecule in normal chromatin states and in the development of the mammalian brain.
A. A Model to Incorporate XCI and MeCP2 Changes During Neuronal Nuclear Maturation

The MeCP2 expression data outlined in Sections III.B and III.C suggest that elevated MeCP2 expression is acquired in CNS neurons during maturational differentiation. This is a time when neuronal size increases and nuclear organizational changes are observed. Neurons become much more transcriptionally active in the process of making dendritic branches and synapses. The model in Fig. 2 proposes a role for MeCP2 in the nuclear changes of neuronal maturational differentiation. Immature fetal nuclei have small heterochromatic nuclei that enlarge slightly with slight increased expression.
in early infancy. The MeCP2lo population observed in the adult brain may simply be immature neurons that have not yet undergone maturation, and thus would be the “default” pathway for neurons that have not been signaled to further differentiate. An activation signal that promotes differentiation may then induce the transcriptional and/or posttranscriptional changes that increase MeCP2 protein expression in individual neurons. Elevated levels of MeCP2 would therefore be required to reorganize the nuclear chromatin to create the phenotypically large euchromatic nuclear structure observed in the MeCP2hi population (Fig. 1A).

In the female RTT brain, approximately half the neurons are MECP2-wild-type expressing (MECP2-wt) and the other half are MECP2-mutant expressing (MECP2-mt), because of random XCI. In this model, mutation of MECP2 would result in the absence of neuronal maturation because of the absence of normal maturational chromatin changes. Therefore, these nuclei would simply be arrested at the immature stage of development when MeCP2 was expressed at a low level and not essential. This would result in all the MECP2 mutant cells having the immature MeCP2lo phenotype. The next part of the model suggests that the MECP2-wild-type expressing cells may be affected in their normal maturational differentiation as well. If neurons in the postnatal CNS require signals from surrounding neurons in order for MeCP2 to become expressed at a high level, many of the MECP2-wild-type expressing neurons may be functionally immature because they are surrounded by other immature neurons that cannot make proper synapses, as the MECP2 mutant cells are randomly distributed in the RTT brain (LaSalle et al., 2001). It may be that a certain percentage of MECP2-mutant expressing cells is required to be present for the effect on wild-type neurons to be manifested, thus explaining why classic RTT girls tend to have random XCI.

This model would therefore revisit the original idea that RTT is a disorder of arrested development (Philippart, 2001). The primary defects observed in RTT brain and MeCP2-null mice may be explained by developmental arrest rather than specific transcriptional changes. MeCP2 would still have a prominent role in chromatin organization, but more as a global regulator of nuclear heterochromatin than as a specific regulator of individual genes. The hypotheses in this model are potentially testable, but the challenge will be in designing experiments that examine MeCP2 in the context of the appropriate tissue and developmental stage.

B. Remaining Questions and Future Directions

Clearly much more work lies ahead in deciphering the complex role MeCP2 plays in normal developing brain and the pathological consequences of RTT. Identifying downstream target genes regulated by MeCP2 is of high
priority. Approaches involving both genome-wide expression profiling and specific targeting of candidate genes need to continue. In addition, understanding the mechanisms underlying the developmentally dynamic expression pattern of MeCP2 seems essential to all future experiments. The promoter elements that regulate MECP2/Mecp2 regulation have not been characterized and would be important in understanding transcriptional regulation of MECP2/Mecp2 in the transition from the MeCP2\textsuperscript{lo} to MeCP2\textsuperscript{hi} cellular phenotype. In addition, even less understood are the sequences and factors required for the developmental control of MECP2/Mecp2 alternate polyadenylation. Could RTT patients without detectable mutations in the coding region of MECP2 be screened for mutations in these regulatory sequences? Could additional genes that encode proteins regulating these processes be mutated as well, resulting in a similar phenotype?

The Mecp2-null and Mecp2\textsuperscript{308/y} mouse models have been extremely helpful, but much more work lies ahead. Do Mecp2 mutant heterozygous females display random or skewed XCI? Can cognitive or behavioral defects be observed at earlier stages in the heterozygous mice? What role does XCI versus Mecp2 genotype play in the development of symptoms and can these findings be extrapolated back to human genotype–phenotype correlations? In addition, culturing CNS neurons from these mice is expected to be especially important in understanding the effect of Mecp2 loss-of-function on the appropriate cell type and stage of differentiation. Gene expression profiling experiments performed on transcripts isolated from neurons induced to differentiate may reveal true targets of MeCP2 regulation rather than downstream effects.

The Mecp2-null mouse models will also be extremely important in designing potential therapies for RTT, but such possibilities also raise many interesting questions. Can wild-type Mecp2 be reexpressed by gene therapy in Mecp2-null mice to ameliorate the symptoms? Will the regulatory control elements of Mecp2 need to be introduced in order to ensure proper developmental expression? Is expression of wild-type Mecp2 in the postnatal brain early enough to prevent symptoms or should wild-type Mecp2 be expressed in fetal development? Because gene therapy approaches in brain have many potential problems, another exciting possibility is to try to specifically activate the wild-type Mecp2 allele on the inactive X chromosome in heterozygous females. Perhaps pharmacological interventions could be designed to try to provide neuronal maturation signals to the Mecp2 wild-type expressing cells in female heterozygotes that may lessen the severity. What role do genetic background and environmental factors play in modulation of the phenotype and can these factors be used in treating RTT?

Another exciting avenue for future work is in understanding the potential importance of MeCP2 in a wider spectrum of neurodevelopmental disorders in addition to RTT. The finding of MECP2 mutations in populations with
mental retardation, Angelman syndrome, and autism suggests that this may be a worthy endeavor (Beyer et al., 2002; Couvert et al., 2001; Lam et al., 2000; Orrico et al., 2000; Watson et al., 2001). Instead of limiting the screen for mutations to the MECP2 coding region, perhaps looking for differences at the level of protein expression in brain samples from multiple neurodevelopmental disorders would be informative. Because of the complex developmental expression of MECP2, mutations may occur in regulatory sequences or in other genes that regulate MECP2. Thus, a seemingly rare genetic disorder such as RTT may serve as an important paradigm for sorting out the genetic and epigenetic mechanisms involved in the complex process of mammalian brain development.

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References


3. Paradoxical Role of MeCP2 in Rett Syndrome


3. Paradoxical Role of MeCP2 in Rett Syndrome


Genetic Approaches to Analyzing Mitochondrial Outer Membrane Permeability

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I. Introduction

Voltage-dependent anion channels (VDACs) are pore-forming proteins found in the mitochondrial outer membrane (MOM) of all eukaryotes (Colombini, 1979; Colombini et al., 1996). VDACs have been commonly referred to as “mitochondrial porins” by analogy to the porins of the outer membrane of gram-negative bacteria (Benz, 1985). VDAC is a voltage-gated protein and, in vitro, the membrane potential is the primary factor in determining the conductance states. Secondary and tertiary structure analyses predict that the VDAC channel adopts a conserved $\beta$-barrel conformation formed by 16 $\beta$ strands reminiscent of bacterial porins (Casadio et al., 2002; Mannella, 1998; Song and Colombini, 1996; Song et al., 1998). The N-terminal globular $\alpha$-helix and putative flexible loop regions are
predicted to face the cytosolic compartment. Although VDACs likely play a central role in the regulated flux of metabolites across the MOM, their exact cellular roles are not well understood.

The existence of multiple related but distinct isoforms suggests that each have distinct functions. Although VDACs are structurally and electrophysiologically highly conserved across species, the specific function of each isoform remains unknown. It has been reported that VDACs associate with the adenine nucleotide translocator (ANT) at the contact sites between the mitochondrial inner and outer membranes (Brdiczka et al., 1998). VDACs may serve as a binding site for cytosolic hexokinases, providing the enzyme with preferential access to ATP derived from oxidative phosphorylation (de Cerqueira Cesar and Wilson, 1998). VDACs are also believed to participate in the phosphocreatine circuit that links sites of energy production to sites of energy consumption, leading to the compartmentation of high-energy metabolites (Wallimann et al., 1992). Although it has been reported that VDACs interact with both antiapoptotic and proapoptotic Bcl-2 family members, whether it is the opening or the closure of VDACs that contributes to the onset of apoptosis (e.g., mitochondrial swelling and cytochrome c release) is still a matter of considerable debate (Tsujimoto and Shimizu, 2002; Vander Heiden et al., 2001).

Given its relative abundance in the MOM, VDAC has long been the subject of in vitro electrophysiological studies. A comprehensive review of the biophysical properties of VDAC in reconstituted or in vitro systems is beyond the scope of this chapter and is reviewed elsewhere (Blachly-Dyson and Forte, 2001; Colombini et al., 1996; Sorgato and Moran, 1993). When VDAC proteins are inserted into planar phospholipid membranes, incremental increases in membrane conductance, clustered at about 4.5 nS, can be recorded. VDACs are slightly anion selective at low voltage and switch to substates characterized by lower conductance at a voltage above 20 mV (Benz, 1985; Colombini, 1979; Roos et al., 1982). The substates of VDACs are cation selective (Ludwig et al., 1988). These different substates have been characterized by using polyanionic compounds, both in intact mitochondria and in vitro lipid bilayers (Benz and Brdiczka, 1992). The structural changes by which VDAC goes from a state of high permeability to one of low permeability are great. The pore diameter that accompanies the “closed” and “open” states is estimated to change from 0.8–0.9 to 1.2–1.5 nm. This is consistent with significant changes in VDAC permeability to the metabolites that cross the mitochondrial outer membrane, for example, pyruvate, succinate, MgADP$, and MgATP$^{2−}$. The induction of impermeable-substates in intact mitochondria would be predicted to lead to a complete inhibition of intermembrane kinases such as creatine kinase and adenylate kinase, which because of their location are excluded from the external ATP pool. It has been proposed that the amino terminus of VDAC regulates the conductive state of the protein (Blachly-Dyson et al., 1989).
Studies on biochemically purified VDACs, either in vitro or in reconstituted systems, do not take into account the potential multiple interactions of VDACs with different cellular components (e.g., cytosolic enzymes or the cytoskeleton). Furthermore, these studies often do not distinguish between VDAC isoforms. The study of phenotypic manifestations of individual VDAC null mutations in genetic model systems provides an important approach in evaluating VDAC function in an in vivo context. The present chapter summarizes reports investigating VDAC-deficient yeast, fruit flies, and mice that provide insights into the physiological function of eukaryotic VDAC isoforms in a cellular and organismal context.

II. VDAC and Saccharomyces cerevisiae

A. Loss of VDAC Results in Respiratory Defect

Studies of the phenotypic consequences of loss of function of VDAC were first performed in yeast. Deletion of the endogenous POR1 (Δpor1) gene by gene disruption resulted in a yeast mutant that exhibited a partial respiratory defect (Dihanich et al., 1987). This partial defect is manifested by the inability to grow on a nonfermentable carbon source (glycerol) at 37°C. In addition, Δpor1 mitochondria were shown to be deficient in cytochromes by spectrophotometry as well as to exhibit a 10-fold reduction in cytochrome c oxidase activity (Dihanich et al., 1987).

B. Identification of POR2 Gene

Because Δpor1 yeast demonstrated only a partial respiratory defect, that is, could grow on glycerol at 30°C but not at 37°C, it was hypothesized that yeast may harbor one or more additional genes that provide at least partial functional redundancy with POR1. A multicopy suppressor screen designed to isolate genes that allow POR1-deficient yeast to grow on glycerol at 37°C was performed and a second VDAC-like gene, POR2, was identified (Blachly-Dyson et al., 1997). The same gene had previously been isolated on the basis of its sequence homology to mouse VDAC isoforms (Sampson et al., 1996b). Comparison of POR1 (also known as YVDAC1) and POR2 (YVDAC2) amino acid sequences revealed 49% identity. Yeast deficient for POR2 (Δpor2) are able to grow on glycerol at both 30 and 37°C, whereas Δpor1Δpor2 yeast, deficient for both POR1 and POR2, demonstrate a much more severe respiratory defect than Δpor1, with poor growth on either glucose or glycerol at any temperature. Whereas overexpression of POR2 functionally complements POR1, purified YVDAC2 protein does not exhibit any
channel-forming activity in reconstituted lipid bilayers (Blachly-Dyson et al., 1997). Although the underlying molecular mechanism of the VDAC-deficient respiratory defect is unknown, this observation underscores the need for caution when extrapolating in vitro data to cellular function and the importance of genetic approaches to study VDAC function in vivo.

C. VDAC-Deficient Yeast as Genetic Tool

The generation of mutant yeast deficient for VDAC has provided a valuable genetic reagent for studying specific VDAC isoforms in vivo. Analysis of mitochondria isolated from Δpor1, Δpor1Δpor2, or Δpor1 with POR2-overexpressed yeast strains demonstrated the requirement of YVDAC1 for MOM permeability to NADH (Lee et al., 1998). Expression of YVDAC1 mutants generated by site-directed mutagenesis in Δpor1 yeast has allowed for elegant structure–function studies in reconstituted systems, leading to the identification of residues important for anion selectivity as well as for voltage gating (Blachly-Dyson et al., 1990; Thomas et al., 1993). Finally, expression of eukaryotic VDACs from other species in Δpor1 yeast has allowed for the comparison of individual VDAC isoforms in terms of in vitro biophysical properties as well as in vivo functional complementation, demonstrating that VDAC functions are conserved across eukaryotes (Blachly-Dyson et al., 1993; Komarov et al., 2003; Sampson et al., 1997; Xu et al., 1999).

III. VDAC and Drosophila melanogaster

A. Multiple VDAC Genes Clustered in Drosophila Genome

Analysis of the annotated Drosophila genome reveals four genes with homology to known eukaryotic VDACs (Reese et al., 2000; Rubin et al., 2000). The four genes are closely linked and exist in tandem on the left arm of chromosome 2 (Fig. 1). These genes include the previously reported porin (Ryser et al., 1997), as well as three previously unrecognized genes (currently designated CG17137, CG17139, and CG17140) (Oliva et al., 2002). Comparison of each gene structure reveals a similar organization with the coding sequence divided into three exons (Fig. 1). porin is notable for an alternatively spliced first exon containing 5′ untranslated region (UTR) sequences (Oliva et al., 1998). Interestingly, the exon/intron boundaries are conserved across the four genes, as well as compared with homologous positions in the mammalian VDAC genes (data not shown; and Oliva et al., 1998). When Drosophila and mammalian VDAC amino acid sequences are analyzed, three levels of homology are apparent: porin exhibits approximately 60%
sequence identity to mouse VDAC1-3; CG17137 exhibits approximately 34% sequence identity to mouse VDAC1-3; and CG17139 and CG17140 exhibit approximately 22–23% sequence identity to mouse VDAC1-3. The predicted proteins encoded by CG17139 and CG17140 are distinct from those encoded by porin and CG17137 in that each contains a large amino-terminal extension (40–60 residues), the function(s) of which remains to be determined. Thus, in summary, it appears that this Drosophila VDAC gene cluster is derived from a series of three separate tandem duplication/divergence events: an ancient duplication from a primordial VDAC gene (an ancestral VDAC gene common to both porin and mammalian VDACs) that gave rise to the precursor of CG17139 and CG17140, and two more recent events that generated CG17137 along with CG17139 and CG17140.

B. Functional Analysis of Drosophila VDACs in Δpor1 Yeast

Previous studies characterizing murine VDACs demonstrated that expression of mammalian VDACs in VDAC-deficient yeast rescues a respiratory-deficient, conditional growth phenotype (i.e., inability to grow on glycerol, a
To begin to better characterize fly VDACs, this same approach was applied to the Drosophila VDAC genes. Yeast shuttle vectors (both centromere and 2μm) that contain the yeast POR1 (YVDAC1) 5′ and 3′ regulatory sequences were utilized (Blachly-Dyson et al., 1990). cDNA for each fly gene was subcloned into the yeast shuttle vector such that its expression in yeast would be controlled by the yeast POR1 regulatory sequences. These vectors were used to transform POR1-deficient yeast, and the ability to grow on glycerol at 30 and 37°C was assessed. porin and CG17137 demonstrate complete complementation either on single copy number (CEN, CG17137) or multiple copy number (2μm, porin) plasmids (Fig. 2).

Electrophysiological characterizations of these proteins in reconstituted systems demonstrate a differential ability to permeabilize lipid bilayers and

![Figure 2](image-url)
C. Genetic Analysis of *porin* Mutants

Oliva *et al.* (2002) reported a study of a series of mutations affecting the level of expression of *porin*. Phenotypic screening was performed on fly strains established from excision of a P element inserted upstream of the *porin* coding sequence [ *porin*(2); Fig. 1](Oliva *et al.*, 2002). Mutants exhibiting recessive lethality in the late pupal stage as well as mutants exhibiting male sterility were identified. Molecular analyses of these mutants revealed a series of deletions upstream of the *porin* coding sequence resulting from imprecise P element excision. Flies from a strain with a *porin* allele resulting from precise excision of the P element (i.e., 5' *porin* genomic sequence identical to wild type) are viable and fertile. Western analysis of protein extracts derived from pupae homozygous for the various deletion alleles demonstrated a range of reduced *porin* expression compared with wild type: from a hypomorphic allele (male sterility) to true null alleles (recessive lethality). Interestingly, these phenotypes are reminiscent of phenotypes observed with null mutations in mouse VDAC isoforms: MVDAC1−/− mice exhibit reduced viability and MVDAC3−/− males are sterile (Sampson *et al.*, 2001; Weeber *et al.*, 2002). Further studies of mutants involving *porin* as well as the other three VDAC-like genes ( *CG17137*, *CG17139*, and *CG17140*) are required to examine the functions of these VDAC-like isoforms in more detail. Mobilization of various P elements localized in the VDAC gene cluster region on chromosome 2 (Fig. 1) should facilitate the generation of additional allelic series of deletions. Preliminary results indicate that deletion mutations involving *CG17140* lead to recessive lethality, reflecting the essential nature of this gene (Graham and Craigen, unpublished results, 2003).

IV. VDAC and *Mus musculus*

A. Genetics of Mammalian VDACs

The genes encoding VDACs have been cloned and sequenced from a number of mammalian species, including human (Blachly-Dyson *et al.*, 1993; Ha *et al.*, 1993), mouse (Sampson *et al.*, 1996a,b), rat (Anflous *et al.*, 1998;
Bureau et al., 1992), and cow (Dermietzel et al., 1994). In mammals, three VDAC genes have been isolated and characterized: *Vdac1*, *Vdac2*, and *Vdac3*. In humans and mice the three isoforms are each encoded by separate autosomal genes (Fig. 3). There are also numerous intronless pseudo-genes for each isoform, one of which in the mouse arose recently enough to still contain an open reading frame. The three VDAC isoforms belong to a single gene family that arose by gene duplication and divergence (Sampson et al., 1997). Phylogenetic analysis indicates that *Vdac1* is the more primordial of the vertebrate VDAC genes, suggesting that as the multiple isoforms arose from gene duplication events *Vdac3* diverged first from the primordial VDAC, with *Vdac2* arising more recently. An additional *Vdac3* isoform is synthesized via alternative splicing of a three-nucleotide exon (ATG) (Anflous et al., 1998; Sampson et al., 1998). The exon introduces a methionine 39 amino acids downstream of the amino terminus of the polypeptide (Fig. 4). Expression of this alternative form appears to be limited to brain, heart, and skeletal muscle. The functional significance of this alternative splicing remains unknown, but its presence in multiple species presumably reflects some highly conserved function. Both mouse *Vdac3* isoforms are able to partially rescue the temperature-sensitive phenotype of

**Figure 3**  Genomic organization of the three mouse VDAC genes. Both *Vdac1* and *Vdac3* are encoded in 9 exons, whereas *Vdac2* is encoded in 10 exons. Open boxes indicate the relative size and location of the different exons (drawn to scale). The translation start and stop sites for each gene are indicated. A Northern blot of each gene, using embryonic stem cell RNA, is shown. Note the presence of multiple *Vdac2* transcripts in comparison with *Vdac1* and *Vdac3*. Figure from Sampson et al. (1997).
VDAC-deficient (Δpor1) yeast, indicating that in yeast the proteins appear to act similarly. However, the biochemical basis for this complementation remains unknown. Interestingly, deletion of the amino-terminal 39 residues from mouse VDAC3 results in a truncated VDAC3 that can, in contrast to the full-length protein, fully rescue the mutant yeast strain. This suggests that the amino-terminal region of VDAC3 has some inhibitory regulatory property.

The human and mouse VDAC amino acid sequences share ~25% similarity with *Saccharomyces* and *Neurospora* VDAC sequences. However, as observed with all eukaryotic VDACs, predictions of the secondary structures demonstrate a greater degree of conservation, with the hydrophobicity plots of each VDAC being highly conserved. Using radiation hybrid analysis, the human genes encoding VDAC1 and VDAC2 have been assigned to chromosomes 5q31 and 10q22, respectively (Decker et al., 1999). The mouse genes encoding VDAC1, VDAC2, and VDAC3 were mapped by means of an interspecies backcross panel to the proximal regions of mouse chromosomes 11, 14, and 8, respectively (Sampson et al., 1996a,b) and maintain the same syntenic relationships to adjacent genes as the human genes. Each gene is composed of 9 or 10 exons contained within less than 17 kilobases of DNA (Fig. 3). The 5′-flanking region of each VDAC isoform contains multiple putative transcription factor-binding sites (Sampson et al., 1997). Commonly, genes expressed in a constitutive fashion are transcribed from...
promoters that contain at least one Sp1-binding site. Mouse \textit{Vdac1} contains 2 Sp1 sites, \textit{Vdac2} contains 10 Sp1 sites, and \textit{Vdac3} contains 5 Sp1 sites. A sterol repressor element 1-binding site is also located in the promoter region of \textit{Vdac1} and \textit{Vdac2}, which suggests a role for sterols in the regulation of \textit{Vdac1} and \textit{Vdac2} expression; however there is no direct evidence for this currently. Interestingly, the \textit{Vdac2} gene contains three polyadenylation sites in the 3′ region. Each site is used to varying degrees, with the most proximal site being the most common site of transcription terminal. The role of these three distinct transcripts is unknown but may reflect a posttranscriptional mechanism for regulating protein levels.

Each VDAC transcript appears to be ubiquitously expressed in mouse tissues (Sampson et al., 1996 a,b). The single exception is \textit{Vdac1}, the mRNA of which cannot be detected by Northern analysis in the testes. However, the expression level of each protein does vary considerably between tissues. In addition, the intracellular distribution of each isoform remains to be determined. It is plausible that mitochondria confined to different subcellular compartments, for example, intermyofibrillar versus subsarcolemmal regions of muscle fibers, maintain a different VDAC composition.

**B. Generation of Mammalian VDAC Mutants**

Embryonic stem (ES) cell lines lacking each VDAC isoform were generated by homologous recombination (Wu et al., 1999). The strategy used to target the \textit{Vdac1} gene was designed to delete exons 2–5. Gene targeting of the \textit{Vdac2} gene was designed to delete the promoter and first two exons, including the predicted start codon. Gene targeting of the \textit{Vdac3} gene deleted the last four of the nine exons of the \textit{Vdac3} gene. All three mutations create a null allele. In each cell line the remaining wild-type allele was subsequently disrupted to generate VDAC-deficient cell lines. On the basis of the significant loss of cytochrome c oxidase (COX) activity in yeast lacking \textit{POR1}, the cell lines were examined for loss of respiration and any reduction in respiratory chain activity. ES cells lacking each isoform are viable but exhibit an ∼30% reduction in oxygen consumption. VDAC1- and VDAC2-deficient cells exhibit a partial reduction in COX activity, whereas VDAC3-deficient cells have normal COX activity. These results indicate that VDACs are not essential for cell viability, and it was speculated that reduced respiration in part reflects decreased outer membrane permeability for small metabolites necessary for oxidative phosphorylation.

VDAC1- and VDAC3-deficient ES cells have been used to generate mouse chimeras and germ line-transmitting mice (Anflous et al., 2001; Sampson et al., 2001; Weeber et al., 2002). \textit{Vdac1} mutant mice are born at a reduced frequency, depending on the mouse strain background. For example, \textit{Vdac1}
deficiency, when bred onto the C57BL/6 strain, is almost completely lethal whereas on a CD1 background mutant mice appear at almost expected frequencies. Thus, this lethal trait is subject to strain-specific modifier gene effects and offers the opportunity to identify those genes that interact with *Vdac1* on a developmental level. Double-mutant mice (*Vdac1/3*) have also been obtained by intercrossing *Vdac1* and *Vdac3* heterozygous mice. Although double-mutant mice also exhibit a partial in utero lethality and are growth retarded, the mice are viable and therefore offer the possibility of studying the role of VDACs in cellular metabolism in intact organisms. Using double-mutant mice allows for the investigation of single-channel function in the absence of other isoforms.

### C. Role of the MOM in Compartmentation of High-Energy Metabolites

The exchange of metabolites between the matrix and the cytosol is a potential site of regulation of mitochondrial function that involves transport across both mitochondrial membranes. Under a variety of conditions *in vitro*, the MOM has been shown to limit the rate of metabolite flux. In parallel, compounds that close VDACs greatly inhibit mitochondrial function by restricting the flux of adenine nucleotides into the mitochondrial intermembrane space (Benz *et al*., 1988; Colombini *et al*., 1987; Gellerich *et al*., 1989; Lee *et al*., 1994; Liu and Colombini, 1992). It has been reported that pyridine dinucleotides decrease the permeability of the mitochondrial outer membrane (Lee *et al*., 1994, 1996). Rostovtseva and Colombini demonstrated that VDAC channels isolated from mitochondria of *Neurospora crassa* and reconstituted into planar phospholipid membranes mediate and gate the flow of ATP in their open state (Rostovtseva and Colombini, 1996, 1997). Channel closure results in 50% reduction in ion conductance, and ATP flux is almost completely blocked. Hence, VDACs can potentially regulate the movement of ATP between the cytosol and the mitochondrial spaces and therefore may participate in controlling ATP flux through the MOM.

Additional evidence implicating VDACs and the regulation of MOM permeability as a critical factor in cellular function and development involved demonstrating a defect in ATP/ADP exchange across the mitochondrial outer membrane after growth factor withdrawal. This was associated with the accumulation of creatine phosphate in the intermembrane space, subsequent loss of MOM integrity, cytochrome *c* release, and apoptosis (Vander Heiden *et al*., 2000). This reduction in outer membrane permeability correlates with the changes in conductance properties that accompany closure of VDACs. In a subsequent report, the authors also demonstrated that the antiapoptotic protein Bcl-xL is able to restore metabolite exchange across the outer membrane without inducing the loss of cytochrome *c* from the intermembrane space,
presumably by maintaining VDACs in an open configuration (Vander Heiden et al., 2001). In a separate report, Le Mellay et al. (2002) have shown that the oncogenic protein c-Raf forms a complex with VDAC in vivo and blocks reconstitution of VDAC channels in planar bilayer membranes in vitro. The authors proposed that this interaction might be responsible for the Raf-induced inhibition of cytochrome c release from mitochondria on growth factor withdrawal. They further speculated that c-Raf kinase-induced VDAC inhibition might regulate the metabolic function of mitochondria and mediate the switch to aerobic glycolysis that is common to cancer cells. It was also proposed that the action of c-Raf and Bcl-2 in blocking cytochrome c release on binding to VDAC might be mediated by two distinct mechanisms: pore reduction versus pore closure (Le Mellay et al., 2002).

VDACs have also been identified as the MOM-binding sites for cytosolic kinases, for example, glycerol kinase and hexokinase (Fiek et al., 1982; Linden et al., 1982). It has been hypothesized that this interaction facilitates access of kinases to ATP generated by oxidative phosphorylation and overcomes the restriction that the MOM exerts on the permeability for small metabolites (Brdiczka and Wallimann, 1994). The mitochondrial isoforms of creatine kinase (mi-CK) and adenylate kinase, located in the intermembrane space, have also been reported to interact with VDACs (Kottke et al., 1991; Savabi, 1994). There is some evidence to suggest that this interaction occurs at the contact sites between the inner and outer mitochondrial membrane (Beutner et al., 1996; Kottke et al., 1991). From this accumulation of in vitro data, a model of VDAC as the predominant site of regulation of MOM permeability and of coordination of energy metabolite compartmentation has evolved (Blachly-Dyson and Forte, 2001).

In an attempt to evaluate the hypothesized regulatory role of VDAC in an in vivo model, the functional coupling of mi-CK and VDACs to mitochondrial respiration has been extensively characterized by using selectively permeabilized skinned fibers prepared from heart and skeletal muscle (Kay et al., 1997; Saks et al., 1995; Veksler et al., 1987, 1995). Studies using this technique, unlike studies using isolated mitochondria, preserve the in vivo structural relationships between mitochondria and the cytoskeleton and examine the total mitochondrial population. The functional coupling of mi-CK to mitochondrial respiration can be determined in the presence of creatine by measuring the mitochondrial sensitivity for MgADP, that is, the apparent $K_m$ for MgADP ($K_m[ADP]$). The $K_m[ADP]$ is high in skinned fibers compared with isolated mitochondria, presumably reflecting the low in vivo permeability of the MOM for MgADP. In the intermembrane space, mi-CK generates phosphocreatine by phosphorylation of creatine, using newly synthesized ATP generated in the matrix. It is speculated that creatine and phosphocreatine cycle between the cytosolic and intermembrane compartments through VDAC, while ADP is recycled back to the matrix via
the adenine nucleotide translocase (ANT) to generate more ATP, leading to a shift in $K_m[\text{ADP}]$ toward lower values. Hence, this functional coupling increases the apparent concentration of ADP in close proximity to ANT, which compensates for the low \textit{in vivo} permeability of the MOM for ADP (Gellerich \textit{et al.}, 1989, 1994). The $K_m[\text{ADP}]$ potentially represents an adaptable mechanism for mitochondrial regulation of cellular metabolism. A 3-fold decrease in the $K_m[\text{ADP}]$ in skinned cardiac fibers in rats fed a creatine analog that competitively inhibits creatine transport across the plasma membrane has been reported (Clark \textit{et al.}, 1994). This decrease in $K_m[\text{ADP}]$ is thought to compensate for the reduced cellular level of creatine and phosphocreatine that would presumably affect the phosphocreatine/creatine shuttle. mi-CK-deficient mice also exhibit a decreased $K_m[\text{ADP}]$ in cardiac and oxidative skeletal muscle fibers (Veksler \textit{et al.}, 1995). This suggests that if the phosphocreatine/creatine shuttle is impaired, ADP rather than creatine plays the main role of diffusible phosphate acceptor in the cells.

To address the question of whether VDAC1 constitutes the main pathway for ADP and creatine transport across the MOM \textit{in vivo}, the $K_m[\text{ADP}]$ for skinned fibers from VDAC1-deficient mouse muscle was measured. An alteration in the MOM permeability for ADP in different muscle types in the absence of VDAC1 was observed (Anflous \textit{et al.}, 2001). In cardiac muscle and glycolytic skeletal muscle (gastrocnemius) an increase in the $K_m[\text{ADP}]$ was demonstrated, which supports the hypothesis that VDAC1 constitutes a pathway for ADP across the MOM. In contrast, oxidative skeletal muscle (soleus) exhibited a decrease in both the $K_m[\text{ADP}]$ and $V_{\text{max}}$, which suggests that, in the soleus, the absence of VDAC1 affects the properties of both mitochondrial membranes. These physiologic changes are accompanied by striking ultrastructural changes in both subsarcolemmal and intermyofibrillar mitochonridia. The cristae become compact and the mitochondria enlarge manyfold (Fig. 5). The muscle type-specific changes also point to the complexity of the role(s) played by VDAC1 in different muscle types. However, the creatine effect on respiration in VDAC1-deficient muscles remained unchanged (Fig. 6). This result suggests that the functional coupling of mi-CK to respiration does not require VDAC1, and that VDAC1 is not required for creatine diffusion across the MOM. Whether the persistence of the functional coupling of mi-CK to respiration in VDAC1-deficient mice is due to functional redundancy among VDAC isoforms or to increased activity of mi-CK needs further study. Similar results have been obtained with VDAC3-deficient cardiac skinned fibers (Anflous and Craigen, unpublished results, 2003).

To gain more insight into the physiologic consequence of altered permeability, VDAC-deficient mice have been subjected to exercise-induced stress testing. Mice lacking both VDAC1 and VDAC3 show a significant reduction in time to fatigue compared with control littermates, whereas the single
mutants do not show a reduction in the time to fatigue, despite a partial respiratory chain defect (Anflous and Craigen, unpublished results, 2003). The question of whether the exercise intolerance in double-mutant mice is due to the absence of compensatory mechanisms that may exist in single-mutant mice needs further study. The fact that VDAC-deficient hearts do not show any sign of hypertrophy, despite structurally aberrant mitochondria (Fig. 7), likely reflects a compensatory mechanism, for example, alterations in the expression of some key metabolic genes, to switch metabolic substrate utilization. Indeed, it has been suggested that metabolic switches are a prerequisite for the successful adaptation of the heart to an altered workload (Taegtmeyer, 2002). Measuring substrate oxidation in perfused control and VDAC-deficient hearts will address this question, and preliminary results indicate that metabolic adaptation occurs, with a switch from fatty acid oxidation to anaerobic metabolism (Anflous and Craigen, unpublished observations, 2003). It has been reported that COX VIaH-deficient mice have impaired left ventricular filling or diastolic dysfunction under maximal cardiac load despite a normal cellular ATP content (Radford et al., 2002). Therefore, evaluation of myocardial systolic and diastolic performance under different working conditions in VDAC-deficient mice will give more insight concerning whether the heart develops subtle phenotypes as a consequence of alterations in the permeability of the MOM.

Figure 5 VDAC1-deficient muscle exhibits proliferation of abnormal mitochondria. Electron microscopic study showing mitochondria from wild-type (A) and VDAC1-deficient (B) gastrocnemius muscle. Figure from Anflous et al. (2001).
Sampson et al. (2001) reported that mice lacking VDAC3 are healthy, but males are infertile. VDAC3-deficient males exhibit normal copulatory behavior, but no pregnancies were observed in a large number of matings. VDAC3-deficient males have normal numbers of sperm per epididymis in comparison with wild-type and heterozygous males, and show no significant differences in the size, weight, or histological features of testes. However, when sperm motility was measured, ~70% of the sperm from wild-type or

**Figure 6** Altered mitochondrial sensitivity for ADP in VDAC1-deficient muscle. (A) apparent \( K_m \) (ADP) in saponin-skinned fibers prepared from wild-type (solid columns) and VDAC1-deficient (open columns) ventricular muscle in the absence and presence of 25 mM creatine (Cr). *\( p < 0.05 \); N.S., no significant difference. (B) \( V_{\text{max}} \) in saponin-skinned fibers prepared from wild-type (solid columns) and VDAC1-deficient (open columns) muscle. Figure from Anflous et al. (2001).

**D. VDAC–Cytoskeletal Interactions**

Sampson et al. (2001) reported that mice lacking VDAC3 are healthy, but males are infertile. VDAC3-deficient males exhibit normal copulatory behavior, but no pregnancies were observed in a large number of matings. VDAC3-deficient males have normal numbers of sperm per epididymis in comparison with wild-type and heterozygous males, and show no significant differences in the size, weight, or histological features of testes. However, when sperm motility was measured, ~70% of the sperm from wild-type or
heterozygous males were motile, whereas only 17% of VDAC3-deficient sperm were motile (Sampson et al., 2001).

The structure of sperm is highly organized, especially the sperm axoneme. Epididymal sperm axonemes have a $9 + 2$ microtubule doublet arrangement typical of cilia. Each microtubule doublet has a corresponding outer dense fiber, all of which are morphologically distinct. Two of the outer dense fibers that are associated with microtubules 3 and 8 terminate within the principal piece and form the longitudinal columns of the axoneme. One can orient cross-sections of axonemes by bisecting the axoneme through the longitudinal columns and two associated microtubules (doublets 3 and 8). This arbitrary boundary asymmetrically divides the remaining doublets of the axoneme, with three doublets on one side and four on the other side. When viewed by electron microscopy, 68% of VDAC3-deficient epididymal sperm axonemes (versus 9% of wild-type axonemes) in cross-section demonstrated some structural aberration, most commonly loss of one outer doublet from the normal $9 + 2$ microtubule doublet arrangement (Fig. 8). In the majority of VDAC3-deficient axonemes, the missing doublet corresponds to the last of the four doublets (doublet 7), reflecting a single recurring defect in the axonemal structure. It has been proposed that loss of doublets 4–7 represents sliding of microtubules during attempted motility, with extrusion of half of the axoneme (Merlino et al., 1991). Images through the midpiece of epididymal

Figure 7  Enlarged mitochondria in VDAC1-deficient ventricles. Electron microscopic study showing intermyofibrillar population of mitochondria from wild-type (left) and VDAC1-deficient (right) ventricular muscle. Figure from Anflous et al. (2001).
sperm demonstrate the same defect in doublet 7. Cross-sections through the distal principle piece of testicular sperm only occasionally revealed structural abnormalities, suggesting that the structural abnormality found in epididymal sperm represents instability of the axoneme rather than a defect.
in assembly. Furthermore, the normal structures found in spermatids within the testis suggest that the defect develops with maturation of sperm in the transition from the testes to the epididymis. Electron microscopy of spermatids in the testes revealed enlarged and abnormally shaped mitochondria along the midpiece, similar to that seen in cardiac and skeletal muscle from VDAC1-deficient mice (Anflous et al., 2001).

When examined by dark-field microscopy, cilia from VDAC3-deficient tracheal epithelial demonstrate a normal 9+2 structural organization and normal ciliary movement. However, when sections were dissected 2 mm below the vocal cords and examined by scanning electron microscopy, reduced numbers of ciliated cells were consistently observed, although no increase in respiratory pathology was reported in the mice. This may reflect a developmental effect of abnormal mitochondrial function. This differential effect on sperm and airway cilia has previously been recognized clinically in infertile men (Escudier et al., 1990).

The dynamic behavior of mitochondria in a cell, including morphological changes and organellar trafficking, is now recognized to be the result of intricate interactions between proteins on the outer surface of mitochondria and various components of the cytoskeleton, including actin filaments, microtubules, and intermediate filaments (Bereiter-Hahn and Voth, 1994; Westermann and Prokisch, 2002). It has previously been reported that in rat brain, VDACs are binding sites for microtubule-associated protein 2 (MAP2; Linden and Karlsson, 1996). Novel proteins have been described from yeast that are localized either to cytoskeletal structures or to the mitochondria themselves (Berger and Yaffe, 1996; Yaffe, 1999). Yeast bearing mutations in these proteins display an aberrant distribution of mitochondria under restrictive growth conditions. Mdm 1 is one of these proteins involved in the distribution of mitochondria and their segregation during cell division. In vitro studies have shown that Mdm 1 protein can form structures reminiscent of intermediate filaments (McConnell and Yaffe, 1992). The structurally abnormal mitochondria in VDAC-deficient muscle and VDAC3-deficient sperm may result from the altered interactions between mitochondria and cytoskeletal elements and/or alterations in proteins that play a role in fission/fusion of mitochondria, rather than being a consequence of a respiratory chain defect, energy production, or calcium signaling.

E. VDAC, Mitochondrial Permeability Transition Pore, and Synaptic Functions

The mitochondrial permeability transition pore (MPTP) is defined as a non-specific, voltage-dependent, and cyclosporin A (CsA)-sensitive pore, permeant to any molecules less than 1.5 kDa in mass (Bernardi, 1999; Halestrap
et al., 2002). The MPTP is hypothesized to be involved in the role of the mitochondrion in Ca\(^{2+}\) homeostasis, necrotic cell death, and apoptosis. It is believed that the MPTP is composed of VDAC in the MOM, the ANT in the inner membrane, and cyclophilin D in the matrix compartment (Halestrap et al., 2002). The MPTP opens under conditions of elevated matrix Ca\(^{2+}\), especially when this is accompanied by oxidative stress and depleted adenine nucleotides. Hence, establishing the specific function, if any, of VDACs in the MPTP and the overall apoptotic process remains a central question.

Given the potential role of the MPTP in calcium homeostasis, the role of VDACs in Ca\(^{2+}\) transport was the subject of one report (Gincel et al., 2001). The authors demonstrated that purified VDAC reconstituted into lipid bilayers or liposomes is highly permeable to Ca\(^{2+}\), possesses Ca\(^{2+}\)-binding sites, and is inhibited by ruthenium red (RuR) (an inhibitor of the Ca\(^{2+}\) uniporter). The authors also showed a correlation between RuR inhibition of Ca\(^{2+}\) accumulation, MPTP opening, and VDAC activity, all of which support the accumulating evidence for VDAC being a component of MPTP. More recently, a connection between mitochondrial hexokinase localization and the antiapoptotic action of the serine/threonine protein kinase B (Akt/PKB) has been reported (Gottlob et al., 2001). It has been proposed that Akt/PKB exerts its antiapoptotic action by preventing closure of VDAC. This interaction inhibits intracellular acidification, mitochondrial hyperpolarization and the decline in oxidative phosphorylation that normally precedes cytochrome c release during apoptosis. By increasing the coupling of glucose metabolism to oxidative phosphorylation via a hexokinase–VDAC interaction, it has been speculated that this pathway may contribute to regulating the MPTP opening.

Synaptic plasticity, a term used to describe alterations in the efficiency of synapses that are believed to underlay learning and memory formation, can be determined by standardized in vitro tests using extracellular recordings of stimulus-induced depolarizations. Paired-pulse facilitation (PPF) is widely regarded as a presynaptic phenomenon caused by residual Ca\(^{2+}\) present in the presynaptic terminal following a depolarization that, in turn, facilitates neurotransmitter release in response to a second depolarization (Wu and Saggau, 1994). Long-term potentiation (LTP), a long-lasting increase in the amplitude of postsynaptic depolarizations, has been correlated with many long-lasting forms of learning and memory (Malenka and Nicoll, 1999), and is believed to represent primarily a postsynaptic phenomenon.

VDAC3-deficient mice were observed to behave abnormally relative to wild-type littermates. This observation led us to hypothesize that VDACs, either through effects on ATP synthesis, calcium signaling, altered MPTP function or some combination of these processes, participate in synaptic plasticity. To determine the role that VDACs may play in learning and synaptic plasticity, Weeber et al. (2002) studied wild-type and VDAC1-,
VADC3-, and VDAC1/3-deficient mice. Using a battery of behavioral tests that quantify associative and spatial learning, it was shown that fear conditioning and spatial learning are disrupted in VDAC-deficient mice, with subtle differences between the different mutant strains in associative learning tasks (Fig. 9). Importantly, there were no sensory or motor deficits identified in any of the mutant strains. This represents the first example of a genetically engineered mouse strain in which a mitochondrial defect leads to learning deficits. In the same report, electrophysiological recordings from the hippocampus demonstrated that deficits in LTP and PPF occur in VDAC1- and VDAC3-deficient brain slices, respectively (Weeber et al., 2002). As would be expected, double-mutant mice exhibited deficits in both measures of synaptic plasticity. Thus, a connection was made between behavior, learning deficits, and abnormal cell-to-cell communication. These results demonstrate that mitochondria play an important role in synaptic function and, further, that each isoform has a unique function in brain synapses. Furthermore, inhibition of the MPTP by CsA in wild-type hippocampal slices reproduced the electrophysiological phenotype of VDAC-deficient mice. Hence, if VDACs are in fact a component of the MPTP, these results suggested for the first time that the MPTP has a dynamic, physiologic role in the central nervous system of mammals, and is not simply a pathologic end point to cellular death.

To further characterize the role of MPTP and VDAC in hippocampal synaptic plasticity and to address the issue of whether mitochondrial calcium buffering in the synapse is altered, Levy et al. (2003) examined the effect of blocking the MPTP on synaptic transmission and plasticity. Using concentrations of CsA low enough not to effect calcineurin activity (a phosphatase of known importance in synaptic function), deficits in synaptic plasticity and an increase in baseline synaptic transmission were observed. The resting Ca$^{2+}$ concentration in presynaptic terminals showed a transient increase immediately on incubation with CsA that correlated with the changes in synaptic plasticity and neurotransmission (Levy et al., 2003). These results suggest that the effect of MPTP blockade is via altered calcium handling. Although it has been reported that VDAC is present in the plasma membrane, where it purportedly is concentrated in caveolae and caveola-related domains (Bathori et al., 1999), on the basis of subsequent studies of purified brain mitochondria, the data from studies of VDAC-deficient brain tissues appear specific to mitochondria. Using an in vitro assay of Ca$^{2+}$ transport in isolated brain mitochondria, Levy et al. (2003) demonstrated that CsA-exposed mitochondria buffer Ca$^{2+}$ more rapidly, presumably trigger a more rapid loss of mitochondrial membrane potential, and also release buffered calcium more rapidly (Fig. 10). This phenomenon, known as calcium-induced calcium release, is believed to underlay the ability of mitochondria to buffer high levels of calcium, for example, elevations associated with plasma
Figure 9  Associative learning impairments in VDAC-deficient mice. Initial training of VDAC mutant mice demonstrates normal freezing activity in response to the association of a mild foot shock and an auditory cue in the presence of a novel context (A). Twenty-four hours after training, mice were reintroduced to the training context (B). Wild-type mice and VDAC1-deficient mice show similar freezing behavior. In comparison, VDAC3- and VDAC1/3-deficient mice exhibit reduced contextual freezing behavior. When compared with control mice, all VDAC-deficient mice display significantly reduced freezing behavior in response to the auditory cue in a novel context (C). *p < 0.05. Figure from Weeber et al. (2002).
membrane depolarization, and to maintain sustained elevations in cytoplasmic calcium. Increased uptake of calcium after blockade of the MPTP suggests the MPTP acts as an efflux pathway for matrix calcium. Similarly,
mitochondria lacking VDAC1 released Ca\(^{2+}\) more rapidly than wild-type mitochondria; however, the initial uptake of calcium was neither as rapid nor as large (Fig. 10E and F). On the basis of these studies it was suggested that the MPTP and VDACs might contribute to the regulated uptake and release of cations from mitochondria to prevent calcium overload and subsequent membrane depolarization. It was also demonstrated that it is not the total Ca\(^{2+}\) uptake but the rate of Ca\(^{2+}\) uptake that appears to play a role in triggering Ca\(^{2+}\) release. VDAC deficiency appears to be detrimental to the proper function of the MPTP because the absence of VDAC1 may result in reduced Ca\(^{2+}\) uptake but an increased likelihood of mitochondrial depolarization and subsequent Ca\(^{2+}\) release.

Whether the alterations in the properties of the MPTP in VDAC-deficient brain is a direct result of the absence of VDACs or is indirectly related to respiratory chain defects detected in VDAC-deficient brain samples (Scaglia and Craigen, unpublished results, 2003) needs further clarification. In contrast to CsA-treated mitochondria, brain mitochondria from VDAC1-deficient mice are impaired in mitochondrial Ca\(^{2+}\) uptake (Fig. 10F). Thus the role of VDACs in Ca\(^{2+}\) flux may not only be as a component of the MPTP but also as a Ca\(^{2+}\) regulator. The overall results highlighted by Weeber et al. (2002) and Levy et al. (2003) point to a dysregulation of resting Ca\(^{2+}\) in the presence of CsA or in the absence of VDACs. Changes in calcium homeostasis may in turn have an impact on signal transduction pathways important in synaptic transmission and subsequent plasticity. Determining the intramitochondrial Ca\(^{2+}\) content at rest and on depolarization in the absence of VDACs will give further insight concerning the involvement of VDACs and MPTP in the dynamic phases of mitochondrial Ca\(^{2+}\) and, indirectly, Ca\(^{2+}\) homeostasis in the cytosol.

**F. VDACs and Apoptosis**

The role of VDACs in apoptosis has remained a controversial topic. Whether VDACs constitute a release pathway into the cytosol or play a role in compartmentation of metabolites is the subject of ongoing debate. Likewise, whether VDACs directly interact with pro- or antiapoptotic proteins has not been unequivocally answered. The multidomain proapoptotic molecules BAK and BAX constitute essential components of the intrinsic death pathway, functioning at the level of both mitochondria and endoplasmic reticulum Ca\(^{2+}\) homeostasis (Wei et al., 2001). On activation, BAX and BAK homooligomerize, resulting, either directly or indirectly, in the permeabilization of the MOM and the release of several proapoptotic proteins, including cytochrome c, endonuclease G, and SMAC/DIABLO, which initiates a caspase cascade leading to apoptosis. BAX and BAK function in parallel in that
apoptosis can still occur in the absence of one or the other, but not in the absence of both. Shimizu et al. demonstrated in vitro that BAX and BAK promote the opening of VDAC and the release of cytochrome c in reconstituted liposomes derived from rat liver mitochondria, whereas the antiapoptotic Bcl-2 member Bcl-xL prevented VDAC opening and cytochrome c release (Shimizu et al., 1999).

Cheng et al. (2003) provided compelling in vivo evidence that VDAC2 is involved in regulating the activity of BAK. By using a combination of protein cross-linking, affinity purification, liquid chromatography and tandem mass spectrometry, the authors showed that BAK interacts with VDAC2 and not VDAC1. It was demonstrated that endogenous BAK, but not BAX, efficiently coprecipitates with hemagglutinin-tagged VDAC2. The active conformation of BAK exhibits more proteolytic susceptibility than the inactive conformation. Furthermore, the absence of VDAC2 increases the susceptibility of BAK to proteolysis, suggesting that VDAC2 regulates the conformation of BAK. By expressing VDAC1 or VDAC2 in BAK- or BAX-deficient embryonic fibroblasts, it was demonstrated that it is VDAC2 that inhibits apoptosis in BAX (but not BAK)-deficient cells on treatment with the apoptosis inducers staurosporine or etoposide. It was also demonstrated that VDAC2 inhibits tBID-induced apoptosis of BAX-deficient cells, but not of BAK-deficient cells.

VDAC2-deficient primary mouse embryonic fibroblasts were generated from chimeric embryos by microinjection of Vdac2−/− ES cells into wild-type blastocysts. These cells have significantly more spontaneous apoptosis in culture and are more sensitive to staurosporine and etoposide. VDAC2-deficient cells treated with staurosporine underwent a partial release of cytochrome c into the cytosol by 3 h and complete release by 5 h, whereas wild-type cells had not initiated any release of cytochrome c over that time. In concert, BAK undergoes oligomerization within 5 h of staurosporine treatment in the absence of VDAC2 but not in wild-type cells. This conformational activation of BAK coincides with complete release of cytochrome c and peak caspase activation. After staurosporine treatment, VDAC2-deficient cells exhibited proteolytic activation of caspases 3 and 7 earlier than did wild-type cells. The responses of wild-type and VDAC2-deficient cells were similar when the extrinsic death pathway was activated by tumor necroses factor α (TNF-α).

It was concluded that in the absence of inhibition by VDAC2, BAK experiences an enhanced allosteric conformational activation resulting in the release of cytochrome c, caspase activation, and mitochondrial dysfunction concordant with an increased susceptibility to apoptotic death (Fig. 11). These results suggest that VDAC2 may have evolved for a specialized function distinct from that of the other VDAC isoforms, and may be a novel target for modulating apoptosis. In contrast, the in vivo kinetics of apoptosis in the absence of
Figure 11 Model for VDAC2 regulation of BAK *in vivo*. Diagrams depict the interaction between VDAC2 and proapoptotic BAK. In the normal cellular state (A), VDAC2 (red trapezoid) interacts with BAK (blue diamond) in the MOM, preventing BAK from homooligomerizing and thereby preventing cytochrome $c$ (black ovals) release and apoptosis. BAK does not interact with the other VDAC isoforms (yellow trapezoid). When the intrinsic apoptotic pathway of the cell is initiated (e.g., after stimulation by staurosporine or etoposide; B), BAK undergoes homooligomerization, thereby directly or indirectly permeabilizing the MOM to cytochrome $c$, activating a caspase cascade and subsequent apoptotic cell death. Cells deficient for VDAC2 (C) are unable to regulate BAK homooligomerization, thus creating a dysregulation of the intrinsic pathway, manifested by increased spontaneous apoptosis as well as increased sensitivity to staurosporine and etoposide. The question mark indicates that the specific molecular nature of the pathway of apoptotic cytochrome $c$ release is currently not clear.
VDAC1 or VDAC3, following a variety of induction protocols, is not significantly different from that of wild-type animals (Decker, Sheiko, and Craigen, unpublished results, 2003). The exact nature of the release pathway for cytochrome c remains to be established, but the most recent results in the literature suggest that a BAX channel (Kuwana et al., 2002) or a less well-characterized “mitochondrial apoptosis-induced channel” (MAC) (Pavlov et al., 2001) may constitute this pathway.

V. Conclusions

The MOM has historically been viewed as a constitutively permeable membrane, with the inner membrane being the site of regulated transport of metabolites involved in coupled respiration. In the past, in vitro studies have led to the hypothesis that VDACs may be involved in the regulation of a number of mitochondrial functions via regulated ion flux. It is becoming increasingly clear that the outer membrane potentially constitutes an additional site for regulation of cellular function and development via control of metabolite flux, binding of various kinases, or through organelle or cytoskeletal interactions. However, extrapolation from the in vitro data to the role that VDACs may play in cell function and development has remained tenuous. For example, although VDACs are sensitive to voltage gating in vitro, direct evidence of voltage gating in vivo is entirely lacking. The utilization of genetic model systems to study VDAC mutants in yeast, flies, mice, and cell lines has provided valuable genetic reagents and data that have begun to bridge the gap that exists between the data obtained from reconstituted systems and in vivo VDAC function.

The generation of yeast deficient for POR1 has provided a model system for comparing in vivo functions of VDACs from various eukaryotic species through complementation, thereby establishing that VDACs from flies, mice, and humans share conserved functions. Analysis of fruit flies deficient for a VDAC isoform reveals phenotypes (reduced viability and male infertility) similar to phenotypes seen in mice deficient for VDAC isoforms, underscoring again the fundamental conserved functions of VDACs across eukaryotic species. The presence of multiple VDAC-like genes, as well as the availability of powerful genetic tools, highlight Drosophila as a promising genetic model system to identify conserved cellular and developmental functions of VDAC.

On isolation, mitochondria adopt a different configuration and lose their connections with cytosolic factors, including the cytoskeleton (Saks et al., 1995). Thus, developing mammalian models for in vivo assessments of function are critical to understanding the roles of VDACs in the cellular context. For example, the data obtained with VDAC3-deficient sperm suggest that
microtubule–VDAC interaction may play a significant role in mitochondrial function and sperm motility. Although the absence of VDAC1 and VDAC3 leads to altered permeability of the outer membrane for ADP in various muscle types, the extent to which the absence of each isoform affects either subsarcolemmal or intermyofibrillar mitochondria is not known at present. Indeed, these two mitochondrial populations exhibit structural and biochemical differences (Cogswell et al., 1993). Hence, future studies will be needed to determine whether the distribution of VDAC isoforms is uniform among different mitochondrial populations. The data obtained from VDAC-deficient mice suggest that, although VDAC1 and VDAC3 may be part of the MPTP, their physiologic function appears distinct, for example, VDAC1 has a more prominent role in postsynaptic LTP whereas VDAC3 deficiency affects presynaptic PPF. These data also provide a potential connection between the MPTP and cell-to-cell communication in the mammalian brain. Given the clear relationship between BAK-mediated apoptosis and VDAC2, the exact role of the other VDAC isoforms in the MPTP remains an unresolved important issue. Thus, even though detailed models of the cellular roles of VDAC have been built on a strong foundation of in vitro data, only through future studies in genetic model systems will the complexity and nuances of VDAC function fully reveal itself.

References


Mitochondrial Dynamics in Mammals

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I. Introduction

Biologists often think of mitochondria as static, kidney bean-shaped organelles that have the mundane chore of providing energy for the cell. However, the mitochondrial population is in fact dynamic (Bereiter-Hahn and Voth, 1994; Rizzuto et al., 1998), and the hundreds of mitochondria in a cell can have a range of morphologies, including small spheres, long tubules, and interconnected tubules. This morphological plasticity is based on the ability of mitochondria to undergo both organelar fusion and fission. These opposing processes are readily visualized in living fibroblasts, where individual mitochondrial tubules continually migrate back and forth along their long axes on radial tracks (Fig. 1). Frequently, two mitochondrial tubules encounter each other and rapidly fuse, typically end to end. Conversely, there are normally an equal number of fission events that resolve a single mitochondrial tubule into two. By regulating the relative rates of fusion and fission, however, the morphology of the mitochondrial population can be dramatically altered, with important consequences for mitochondrial function.

Here we review our current molecular understanding of mitochondrial fusion and fission. Several good reviews are available for yeast mitochondrial dynamics (Hermann and Shaw, 1998; Jensen et al., 2000; Shaw and
This chapter pays special attention to how these processes might be important in vertebrates. In particular, we discuss the role of mitochondria dynamics in mammalian mitochondrial morphology, mitochondrial function, disease, embryogenesis, and apoptosis.

II. The Central Molecular Players

Significant progress has been made in identifying molecules central to mitochondrial fusion and fission (Table I). The first major discovery was made with the identification of Fzo in Drosophila, and much of the later progress has been made in yeast, where powerful genetic screens have identified several key molecules. Some additional promising molecules have been identified but are beyond the scope of this chapter (Dimmer et al., 2002; Fritz et al., 2003; Messerschmitt et al., 2003).

A. Fusion Pathway

1. Fzo/Mfn

The first central molecule in the control of mitochondrial dynamics was discovered through the study of spermatogenesis in Drosophila (Hales and Fuller, 1997). Ultrastructural studies documented that during spermatid differentiation in Drosophila, mitochondria progress through an unusual but precise set of morphological changes. After meiosis, the mitochondria in spermatids aggregate and fuse into two giant mitochondria that then wrap around each other to form the Nebenkern structure (Fuller, 1993). A

Figure 1 Mitochondrial fusion in mouse embryonic fibroblasts. Time-lapse confocal microscopy was used to image living fibroblasts expressing yellow fluorescent protein targeted to the mitochondrial matrix. The still frames show two pairs of mitochondria, pseudo-colored blue, moving toward each other along their long axes (left), approaching (center), and then fusing (right). Reproduced from the Journal of Cell Biology, 2003, 160(2), p. 193 by copyright permission of the Rockefeller University Press (Chen et al., 2003). Videos showing the full movie, including fission events, can be viewed at http://www.jcb.org/cgi/content/full/jcb.200211046/DC1.
cross-section of this Nebenkern structure, when examined by electron microscopy, resembles an onion slice due to the concentric layers of wrapped mitochondria. The *Drosophila fuzzy onions* (*fzo*) mutant has a specific defect in fusion of mitochondria such that the mitochondria undergo movements like aggregation, but fail to fuse (Hales and Fuller, 1997). As a result, multiple mitochondria remain in the spermatid, resulting in an obvious morphological aberration by electron microscopy. A functional defect evidently accompanies this structural change, because the mutant flies are sterile.

*Fzo* encodes a large transmembrane GTPase, localized to the outer mitochondrial membrane, and is conserved in more complex eukaryotes (Mozdy and Shaw, 2003). The amino terminus of the protein contains a GTPase domain with canonical G1 to G4 motifs. Mutation of the GTPase domain results in a nonfunctional protein that fails to rescue mutant flies (Hales and Fuller, 1997). The transmembrane domain is located toward the carboxyl terminus of the protein and is flanked by two regions with hydrophobic heptad repeats, sequences characteristic of coiled coils (Fig. 2). The presence of coiled-coil and GTPase domains is intriguing, because both of these sequence motifs play important roles in membrane fusion in other systems.

### Table 1  Molecules Involved in Mitochondrial Fusion and Fission

<table>
<thead>
<tr>
<th>Mammalian protein</th>
<th>Yeast protein</th>
<th>Process</th>
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<tbody>
<tr>
<td>Mfn1&lt;sup&gt;a&lt;/sup&gt;, Mfn2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fzo1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Fusion</td>
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<tr>
<td>OPA1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mgm1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Fusion</td>
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<tr>
<td>Unknown</td>
<td>Ugo1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Fusion</td>
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<tr>
<td>Drp1&lt;sup&gt;g&lt;/sup&gt;/DLP1&lt;sup&gt;h&lt;/sup&gt;/DVLP&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Dnm1&lt;sup&gt;j&lt;/sup&gt;</td>
<td>Fission</td>
</tr>
<tr>
<td>Unknown</td>
<td>Mdv1&lt;sup&gt;k&lt;/sup&gt;/Fis2&lt;sup&gt;l&lt;/sup&gt;/Gag3&lt;sup&gt;m&lt;/sup&gt;/Net2&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Fission</td>
</tr>
<tr>
<td>hFis1&lt;sup&gt;o&lt;/sup&gt;</td>
<td>Fis1&lt;sup&gt;p&lt;/sup&gt;/Mdv2&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Fission</td>
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<sup>a</sup>Santel *et al.* (2003); Legros *et al.* (2002); Chen *et al.* (2003).
<sup>b</sup>Santel and Fuller (2001); Rojo *et al.* (2002); Chen *et al.* (2003).
<sup>c</sup>Hermann *et al.* (1998); Rapaport *et al.* (1998).
<sup>d</sup>Delettre *et al.* (2000); Alexander *et al.* (2000).
<sup>e</sup>Shepard and Yaffe (1999); Wong *et al.* (2000).
<sup>f</sup>Sesaki and Jensen (2001).
<sup>g</sup>Smirnova *et al.* (1998); Smirnova *et al.* (2001).
<sup>h</sup>Pitts *et al.* (1999).
<sup>i</sup>Shin *et al.* (1999).
<sup>j</sup>Bleazard *et al.* (1999); Sesaki and Jensen (1999).
<sup>k</sup>Tieu and Nunnari (2000).
<sup>l</sup>Moody *et al.* (2000).
<sup>m</sup>Fekkes *et al.* (2000).
<sup>n</sup>Tieu and Nunnari (2000).
<sup>o</sup>James *et al.* (2003); Yoon *et al.* (2003).
<sup>p</sup>Mozdy *et al.* (2000).
<sup>q</sup>Tieu and Nunnari (2000).
Coiled coils in envelope glycoproteins of certain viruses play a direct role in membrane fusion, including those of the retrovirus, orthomyxovirus, and paramyxovirus families (Eckert and Kim, 2001; Skehel and Wiley, 1998). In intracellular vesicular trafficking, SNARE complexes form similar helical bundles that likely mediate vesicle fusion. Rab GTPases are known to regulate vesicular fusion (Mellman and Warren, 2000). It remains to be determined whether Fzo and its homologs act directly as fusogenic molecules (analogous to viral fusion proteins and SNAREs), or as regulators (analogous to small G proteins), or both.

The budding yeast homolog, Fzo1, also controls mitochondrial fusion. Deletion of FZO1 results in fragmented, spherical mitochondria instead of the normal tubules observed in wild-type cells (Hermann et al., 1998; Rapaport et al., 1998). In addition, mating-induced mitochondrial fusion is defective (Hermann et al., 1998). Subsequent to the fragmentation of mitochondria, fzo1Δ yeast lose mitochondrial DNA (mtDNA) and become

![Figure 2](image_url)  
**Figure 2** Molecules involved in mitochondrial fusion and fission. Schematic of proteins: Mfn1 (mouse, 742 residues), Mfn2 (mouse, 758 residues), OPA1 (mouse, 960 residues), Ugo1 (yeast, 502 residues), Drp1 (mouse, 712 residues), Fis1 (mouse, 250 residues), and Mdv1 (yeast, 714 residues). Charged residues in the transmembrane segments (TM) of Mfn1 and Mfn2 are indicated by white, vertical lines. In contrast to yeast Mgm1, murine OPA1 has no obvious TM region, and it is unknown whether processing involves cleavage by rhomboid-like proteases. HR, Hydrophobic heptad repeats identified by the program MULTICOIL (Wolf et al., 1997); MTS, mitochondrial targeting sequence; METM, mitochondrial energy transfer motif; Central, homologous to dynamin central domain; GED, GTPase effector domain.
deficient in respiration. The mechanism of mtDNA loss is unknown, and is not observed in Mfn-deficient mammalian cells (Chen et al., 2003).

Mammals have two homologs of Fzo, termed mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2). These proteins are 80% similar to each other and are broadly expressed in a wide range of cell types (Rojo et al., 2002; Santel et al., 2003). Most studies have described a uniform localization of human Mfn1 and Mfn2 to the mitochondrial outer membrane (Rojo et al., 2002; Santel and Fuller, 2001; Santel et al., 2003), but one report describes a predominantly punctate localization with lower levels throughout the mitochondria (Karbowski et al., 2002). Murine Mfn1 and Mfn2 are clearly localized in a uniform manner to mitochondria (Chen et al., 2003).

Overexpression studies of human mitofusins have been difficult to understand fully. Overexpression of either human Mfn1 or Mfn2 leads predominantly to perinuclear clustering of mitochondria (Legros et al., 2002; Rojo et al., 2002; Santel and Fuller, 2001; Santel et al., 2003). In the case of Mfn2 overexpression, mitochondrial clustering is not dependent on a functional GTPase (Santel and Fuller, 2001). However, the formation of an interconnected tubular network, through coexpression of Mfn2 with an inhibitor of fission, does depend on a functional GTPase domain (Santel and Fuller, 2001). In the case of Mfn1 overexpression, one study found only perinuclear clustering at high expression levels (Legros et al., 2002). In cells with lower levels of expression, long and interconnected mitochondrial tubules emanated from the perinuclear clusters. In a second study, overexpression of Mfn1 induced the formation of “grapelike” perinuclear clusters (Santel et al., 2003). A GTPase mutation reduced, but did not abolish, the formation of such clusters.

For both Mfn1 and Mfn2, the significance of their ability to cause perinuclear clustering of mitochondria is unclear. Because this effect is not strictly GTPase dependent, it is not necessarily related to their mitochondrial fusion activity, which is known to depend on a functional GTPase domain (Chen et al., 2003). It should be noted that overexpression of various mitochondrial membrane proteins, unrelated to fusion, has been reported to nonspecifically cause mitochondrial clustering or aggregation (Yano et al., 1997). On the other hand, it is possible that the mitochondrial clustering phenotype reflects a physiological function of mitofusins in mediating mitochondrial adherence, a necessary step in progression to full fusion.

Definitive evidence for the functional roles of Mfn1 and Mfn2 has come from loss-of-function analyses. Disruption of Mfn1 or Mfn2 function results in fragmentation of mitochondria in mouse embryonic fibroblasts, secondary to a severe reduction in mitochondrial fusion (Chen et al., 2003). Loss of either Mfn1 or Mfn2 leads to midgestation embryonic lethality. Furthermore, simultaneous loss of Mfn1 and Mfn2 leads to a significantly earlier lethality and more severe developmental delay, indicating
that these related proteins probably act in concert or have some redundant functions.

Interestingly, the mitochondrial phenotypes of cells lacking Mfn1 differ significantly from that of cells lacking Mfn2 (Chen et al., 2003). Cells lacking Mfn1 contain mitochondria that appear as short tubules or small spheres. In contrast, cells lacking Mfn2 have a less uniform mitochondrial population, with both large and small mitochondrial spheres. The mitochondria in both of these mutant cells have aberrations in mitochondrial motility, a phenotype thought to be secondary to the morphological defects. These observations show that Mfn1 and Mfn2 have related but distinct roles in controlling mitochondrial morphology.

In contrast to human mitofusins, overexpression of murine Mfn1 or Mfn2 does not lead to perinuclear clustering (Chen et al., 2003). In addition, overexpression in mouse embryonic fibroblasts does not extend the length of mitochondrial tubules or increase their interconnectivity. However, when the corresponding mitofusin is expressed in mutant cells lacking Mfn1 or Mfn2, the fragmented mitochondria are dramatically restored to long tubules. As expected, this activity requires a functional GTPase domain.

Protease sensitivity experiments on yeast Fzo1 (Fritz et al., 2001) and human Mfn2 (Rojo et al., 2002) indicate that both the amino and the carboxyl termini are oriented toward the cytosol. This membrane topology is supported by analysis of protein fragments expressed in mammalian cells. An Mfn2 fragment, consisting of the transmembrane segment to the carboxyl terminus, contains sufficient information to localize to mitochondria (Rojo et al., 2002; Santel and Fuller, 2001). The carboxyl terminus of this fragment is oriented toward the cytosol, because it is able to interact with a soluble amino-terminal fragment, thereby changing the localization of the amino-terminal fragment from the cytosol to the mitochondria (Rojo et al., 2002).

This topological arrangement indicates that the transmembrane segment must span the outer mitochondrial membrane twice. Indeed, the transmembrane segment has unusual sequence features (Fig. 2). In all Fzo homologs, the transmembrane segment is divided into two or more hydrophobic segments separated by charged residues. This feature has led to suggestions that the two hydrophobic segments each span the outer membrane, with the intervening charged residues located in the intermembrane space, where they may be in a position to interact with intermembrane or inner membrane proteins (Fritz et al., 2001; Santel and Fuller, 2001). This model, however, must accommodate the fact that some of the hydrophobic segments are only 13–15 residues long, shorter than most transmembrane segments that span the membrane as \( \alpha \) helices.

Mitofusins probably function as higher order oligomers. Immunoprecipitation experiments clearly show that both Mfn1 and Mfn2 can physically
interact homotypically or heterotypically (Chen et al., 2003). In mouse fibroblasts, both Mfn1 and Mfn2 have important roles in promoting mitochondrial fusion, because disruption of either protein leads to severe mitochondrial fragmentation. However, these defects can be rescued by overexpression of Mfn1 or Mfn2, showing that either protein is functional as a homotypic oligomer. These results suggest a model in which Mfn1 homotypic oligomers, Mfn2 homotypic oligomers, and Mfn1–Mfn2 heterotypic oligomers are all functional complexes that play different roles in maintaining mitochondrial fusion, depending on the cell type.

2. Mgm1/OPA1

Yeast mutants of the dynamin-related GTPase, Mgm1, behave similarly to fzo1Δ mutants (Shepard and Yaffe, 1999; Wong et al., 2000). They show mitochondrial fragmentation with loss of mtDNA and an inability to survive on nonfermentable carbon sources. These phenotypes are rescued by the concurrent loss of Dnm1, a protein required for mitochondrial fission (Wong et al., 2000). In addition, mgm1Δ mutants are deficient in mating-induced mitochondrial fusion. Interestingly, mgm1Δ yeast contain ultrastructural alterations in the mitochondrial inner membrane (Sesaki et al., 2003), raising the possibility that the fusion defects may be secondary to structural changes in the mitochondria. However, mgm1Δ dnm1Δ yeast contain mitochondrial tubules that do not have such inner membrane alterations, but nevertheless cannot fuse (Sesaki et al., 2003; Wong et al., 2003). Therefore, the fusion defect is most likely the primary deficiency.

Mgm1 is a mitochondrially localized protein with an amino-terminal mitochondrial targeting sequence. The precise subcellular localization of Mgm1 has been controversial, however, with reports attributing it to the mitochondrial outer membrane (Shepard and Yaffe, 1999), intermembrane space with associations with the inner membrane (Wong et al., 2000), or intermembrane space with associations with both the outer and inner membranes (Sesaki et al., 2003). Part of the discrepancy may be due to an unusual proteolytic processing event during maturation of Mgm1. After import into mitochondria, the mitochondrial targeting sequence is removed in the matrix by the mitochondrial processing peptidase (MPP). Then the rhomboid-related transmembrane protease Mdm37/Pcp1/Rbd1 (Herlan et al., 2003; McQuibban et al., 2003) cleaves off a segment near the amino terminus. The region of Mgm1 removed by Mdm37/Pcp1/Rbd1 contains a putative transmembrane anchor, and thus the uncleaved form of Mgm1 may be integrated into a mitochondrial membrane, whereas the mature form may be peripherally associated with a membrane or simply soluble in the intermembrane space.

As with Mgm1, the subcellular localization of its mammalian homolog, OPA1, is unclear. Studies have generally indicated localization to the
intermembrane space, but reports vary as to which membrane it contacts and whether it is integrally embedded in the membrane (Olichon et al., 2002; Satoh et al., 2003). To complicate matters, OPA1 encodes eight splicing variants (Delettre et al., 2001), the subcellular localizations of which may vary (Satoh et al., 2003). Expression studies in mice and humans show that whereas OPA1 can be found in all tissues, expression levels of specific isoforms differ (Delettre et al., 2001; Misaka et al., 2002). All isoforms contain two predicted coiled-coil domains, one toward the amino terminus and one at the extreme carboxyl terminus (Fig. 2). Alternatively spliced exon 5b also encodes a predicted coiled coil immediately prior to the first universal coiled coil.

The precise function of OPA1 in regulating the morphology of mammalian mitochondria remains to be determined. As expected, RNA interference (RNAi)-mediated knockdown of OPA1 leads to fragmentation of the mitochondrial network (Chen and Chan, unpublished results, 2003; Olichon et al., 2003). However, overexpression of OPA1 also changes the normally tubular network of mitochondria into small spheres (Misaka et al., 2002). This phenotype could result from either an induction of mitochondrial fission or an inhibition of fusion. The GTPase dependence of this phenomenon is unclear. Overexpression of various GTPase mutants leads to alterations in morphology, but the biochemical effects of these mutations on GTPase activity have not been tested (Misaka et al., 2002; Satoh et al., 2003). These observations are difficult to fully reconcile without further experiments, but they do suggest that the cellular levels of OPA1 are critical for maintenance of tubular mitochondrial morphology. Either overexpression or underexpression of OPA1 can lead to fragmentation.

3. Ugo1

To identify additional components of the yeast mitochondrial fusion pathway, a genetic screen was used to isolate genes with properties similar to that of FZO1 (Sesaki and Jensen, 2001). In fzo1 mutants, mtDNA is lost, resulting in lack of respiratory competence. However, this loss of mtDNA is prevented if Dnm1 activity has been previously disrupted. In a screen for mutants that show this pattern of Dnm1-dependent loss of mtDNA, UGO1 (as well as FZO1, MGM1, and UGO2) was identified. Like fzo1Δ or mgm1Δ mutants, ugo1Δ mutants show fragmentation of mitochondria, loss of mtDNA, and lack of mating-induced mitochondrial fusion. Biochemically, Ugo1 behaves as an integral protein embedded within the outer mitochondrial membrane (Fig. 2). On the basis of immunoprecipitation experiments, Ugo1, Mgm1, and Fzo1 appear to be components of a large fusion apparatus (Sesaki et al., 2003; Wong et al., 2003). However, only a minority of these proteins appears to coassemble into a complex, implying that the associations are weak or transient.
Unlike Fzo1 and Mgm1, there are thus far no known mammalian homologs of Ugo1. Aside from the transmembrane segment, the only notable sequence feature of Ugo1 is the six-residue sequence motif PX(D/E)XX(K/R) (Sesaki and Jensen, 2001), which is found in mitochondrial carrier proteins such as the ATP/ADP carrier and may be involved in mediating electrostatic interactions (Nelson et al., 1998).

B. Fission Pathway

1. Dnm1/Drp1

Drp1 controls mitochondrial fission in mammalian cells (Smirnova et al., 2001). This dynamin-related protein contains regions homologous to the amino-terminal GTPase domain, central region, and carboxyl-terminal GTPase effector domain (GED) of dynamin (Fig. 2). Much of Drp1 exists as a cytosolic pool, but a fraction localizes to spots on mitochondria that coincide with future fission sites. Overexpression of a dominant-negative variant, Drp1 (K38A), results in collapse of the mitochondrial network into a large perinuclear mass (Pitts et al., 1999; Smirnova et al., 1998). These perinuclear masses are actually congregations of abnormally long and interconnected mitochondria, which can be more easily visualized by dispersing the masses with the microtubule depolymerizing drug nocodazole (Smirnova et al., 2001). Overexpression of dominant-negative Drp1 (Chen et al., 2003) or RNAi directed against Drp1 (Chen and Chan, unpublished results, 2003) can also rescue the mitochondrial fragmentation observed in Mfn1- or Mfn2-deficient cells.

As with dynamin (Danino and Hinshaw, 2001), two fundamentally different functions have been proposed for Drp1. Drp1 may act as a mechano-chemical enzyme driven by GTP hydrolysis to directly mediate membrane fission. Alternatively, it may act as a signaling molecule to regulate the activity of a separate fission machinery. Consistent with the first model, purified Drp1 can assemble under low-salt conditions into higher order structures that appear as rings (Smirnova et al., 2001) and can tubulate liposomes (Yoon et al., 2001), suggesting that Drp1 may mediate fission by forming constricting rings around mitochondria. Support for the second model has come from analysis of the GED domain of Dnm1, the yeast Drp1 homolog (Fukushima et al., 2001).

Disruption of Dnm1 in yeast results in netlike mitochondria (abnormally interconnected networks) that are thought to result from lack of mitochondrial fission (Bleazard et al., 1999; Sesaki and Jensen, 1999). Consistent with this function, loss of \( \text{Dnm1} \) prevents the mitochondrial fragmentation observed in yeast fusion mutants (\( \text{fzo1}\Delta, \text{mgm1}\Delta, \) and \( \text{ugo1}\Delta \)), indicating
that manipulation of the relative rates of fusion and fission can control the overall morphology of mitochondria (Bleazard et al., 1999; Sesaki and Jensen, 1999, 2001; Wong et al., 2000). In Caenorhabditis elegans, over-expression of dominant-negative Drp1 blocks only fission of the outer mitochondrial membrane, while fission of the inner membrane proceeds (Labrousse et al., 1999).

2. Mdv1

Two other components of the fission pathway, Mdv1 and Fis1*, were identified as extragenic suppressors of yeast fusion mutants (Cerveny et al., 2001; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000). Like dnm1 mutants, these fission mutants result in the formation of mitochondria with netlike morphology, presumably caused by lack of mitochondrial fission.

MDV1 encodes a soluble protein with a coiled-coil motif and WD repeats in the carboxyl terminus (Fig. 2) (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000). Remarkably, Mdv1 localizes to mitochondria in punctate spots that also contain Dnm1. Consistent with this colocalization, Mdv1 and Dnm1 have been shown to physically interact by two-hybrid assays (Tieu and Nunnari, 2000; Uetz et al., 2000). In dnm1/C1 mutants, Mdv1 is no longer localized to punctate spots, but instead is found uniformly associated with the mitochondrial membrane (Cerveny and Jensen, 2003; Tieu et al., 2002). Further analysis indicates that the WD repeat region of Mdv1 is sufficient to mediate this colocalization with Dnm1 (Cerveny and Jensen, 2003; Tieu et al., 2002). Thus far, no mammalian homologs of Mdv1 have been identified.

3. Fis1/hFis1

Fis1 is a mitochondrial outer membrane protein required for mitochondrial fission (Mozdy et al., 2000). It is localized uniformly on mitochondria. In fis1Δ cells, Dnm1- and Mdv1-positive mitochondrial spots are still present, but their numbers are dramatically decreased (Mozdy et al., 2000; Tieu and Nunnari, 2000). In addition, a higher proportion of Mdv1 appears to be dispersed in the cytosol. These observations suggest that Fis1 regulates the formation of these mitochondrial structures, which are potential sites of fission.

Fis1 is a small protein with a single transmembrane segment located near the carboxyl terminus (Fig. 2). The majority of the protein faces the cytosol. By both two-hybrid and immunoprecipitation analyses, the cytosolic region

* For clarity, the “standard name” listed in the Saccharomyces Genome Database (www.yeastgenome.org) is used in the text; equivalent gene names are listed in Table I.
of Fis1 physically interacts with Mdv1, thus recruiting it to the mitochondria (Cerveny and Jensen, 2003; Tieu et al., 2002).

Overexpression of human Fis1, termed hFis1, causes fragmentation of the mitochondrial network (James et al., 2003; Yoon et al., 2003). Consequently, it has been proposed that the number of hFis1 molecules present on the mitochondria regulates the amount of fission activity. This fission activity is Drp1 mediated because it is blocked by expression of dominant-negative Drp1. A physical interaction between Drp1 and hFis1 has been demonstrated by fluorescence resonance energy transfer and immunoprecipitation experiments (Yoon et al., 2003). Interestingly, overexpression of hFis1 is also associated with an increase in apoptosis (James et al., 2003).

III. Mitochondrial Dynamics in the Control of Mammalian Mitochondrial Morphology

Several observations indicate that mitochondrial dynamics plays a significant role in vertebrate cells. Time-lapse observations of mammalian cells reveal frequent and constant cycles of mitochondrial fusion and fission (Fig. 1) (Bereiter-Hahn and Voth, 1994; Rizzuto et al., 1998). Cell cycle-dependent changes in mitochondrial morphology have been reported, with reticular structures present in G1 and fragmented structures in S phase (Barni et al., 1996; Margineantu et al., 2002). In artificially fused cells, mitochondrial fusion has been documented. When murine fibroblasts or HeLa cells are fused with polyethylene glycol (PEG) or a hemagglutinating virus, the resulting cell hybrids show intermixing of mitochondrial matrix contents, providing definitive evidence of mitochondrial fusion (Chen et al., 2003; Hayashi et al., 1994; Ishihara et al., 2003; Legros et al., 2002; Mattenberger et al., 2003). The fusion of mitochondria requires an intact mitochondrial membrane potential, and dissipation of this potential results in fragmentation (Ishihara et al., 2003; Legros et al., 2002; Mattenberger et al., 2003). By morphological criteria, mitochondrial fusion is largely completed by 8–12 h after cell fusion (Chen et al., 2003; Legros et al., 2002). Functionally, however, efficient complementation of gene products does not occur until many days later (Ono et al., 2001).

The identification of molecules involved in the fusion and fission pathways has allowed an assessment of their relative roles in controlling mitochondrial morphology. It is clear that inhibition of mitochondrial fission, by overexpression of a dominant-negative mutant of Drp1, results in a greater interconnectivity of the mitochondrial network and an elongation of the length of the mitochondrial tubules (Smirnova et al., 2001). It is also clear that partial inhibition of the fusion pathway, by deletion of either Mfn1 or Mfn2,
results in dramatic shortening of mitochondrial tubules, such that most mitochondria appear as short rods or spheres (Chen et al., 2003). Taken together, these results indicate that the overall mitochondrial network observed in mammalian cells is quite labile. Both fusion and fission must be highly active, and even partial disruption of one process can shift the delicate balance and dramatically alter mitochondrial morphology (Fig. 3).

As expected, then, a defect in the fusion pathway can be at least partially rescued by a compensatory inhibition of the fission pathway. Mitochondrial tubules can be restored to Mfn1- or Mfn2-deficient cells by overexpression of dominant-negative Drp1 (Fig. 3) (Chen et al., 2003). These results present an interesting conundrum. If mitochondrial tubules can be maintained by downregulating both the fusion and fission pathways, why have cells developed such sophisticated machineries to maintain high levels of fusion and fission? The answer must be that a dynamic mitochondrial population has advantages over a static one.

IV. Mitochondrial Dynamics and Mitochondrial Function

What specific advantages a dynamic mitochondrial population has are still not certain, but evidence suggests that fusion promotes intermitochondrial cooperation and fission enables compartmentalization. In particular, fission may allow equitable distribution of mitochondria to the daughter cells during cell division, perhaps explaining the cell cycle dependence of mitochondrial morphology in mammalian cells (Barni et al., 1996; Margineantu et al., 2002). It should be noted, however, that fission is not required for mitochondrial inheritance in yeast, because mitochondrial fission mutants are viable (Bleazard et al., 1999; Sesaki and Jensen, 1999). Fission most likely also allows mitochondria in different parts of the cell to perform discrete functions. For example, mitochondria in different regions of the cell show functional heterogeneity, as assayed by membrane potential, calcium sequestration, and membrane permeability (Collins et al., 2002). On the other hand, fusion may facilitate the rapid transmission of membrane potential (Skulachev, 2001) or the exchange of mitochondrial contents. These contents could include both membrane-bound or diffusible molecules, substrates or products such as ATP. In addition, mtDNA exchange between mitochondria would raise the possibilities of complementing mtDNA mutations, or repairing them through gene conversion.

The ability to experimentally test for mtDNA exchange was enabled by the development of cell fusion techniques that allow the creation of cybrids, hybrid cells containing one nucleus but two populations of mitochondria (King and Attardi, 1989). Mitochondria containing a recessive mtDNA
Figure 3 Relative rates of fusion and fission control the morphology of mitochondria. Wild-type fibroblasts have a tubular mitochondria with high rates of fusion and fission. Inhibition of fission (by dominant-negative Drp1) results in more elongated and interconnected tubules, whereas inhibition of fusion (by disruption of Mfn1 or Mfn2 or by RNAi-mediated reduction of OPA1) results in fragmentation. In cells with reduced fusion, subsequent inhibition of fission can restore mitochondrial tubules (far right).
Mutation can be introduced into a cell line carrying mitochondria with a separate mtDNA mutation, and complementation can be assessed by growth on medium that selects for respiration-competent cells. In one study, only about 1% of the cybrids exhibited complementation (Enriquez et al., 2000). Because mitochondrial fusion results in mitochondria simultaneously carrying both mtDNAs, it was expected that high rates of mitochondrial fusion would lead to efficient complementation. The low levels of complementation observed led to the conclusion that mitochondria (at least in the 143B nuclear background, in which these experiments were performed) are largely autonomous organelles that either fuse rarely or fuse without mixing of mtDNA products.

Analogous experiments using different cell lines yielded different results (Ono et al., 2001). In this case, it was observed that formation of cybrids carrying two different recessive mtDNA mutations resulted in highly efficient restoration of respiratory competence. Intriguingly, the cybrids did not regain respiratory competence until 10–14 days after cell fusion. It is unclear why complementation would take such a long time, given that other experiments show fusion of mitochondria occurring within either minutes (Hayashi et al., 1994) or hours (Chen et al., 2003; Legros et al., 2002; Mattenberger et al., 2003) after cell fusion. The possible reasons for the discrepancy between the two studies have been discussed (Attardi et al., 2002; Hayashi et al., 2002), and may involve an unanticipated lag time needed for restoration of respiratory activity or the effect of nuclear genes. Nevertheless, it appears clear that, in at least some cultured cells, extensive fusion of mitochondria does occur, and this fusion protects mitochondrial function.

The same concept is supported by analysis of cells lacking mitofusin function. In either Mfn1- or Mfn2-deficient cells, a subset of mitochondria loses membrane potential, even though the bulk culture is competent for respiration (Chen et al., 2003). Therefore, in the absence of mitochondrial fusion, the mitochondria are forced to be autonomous, and transient losses of membrane potential can become prolonged or permanent. In spite of rapid mitochondrial fusion, however, it should not be assumed that the entire mitochondrial population is necessarily homogeneous. At any given time, individual mitochondria are electrically uncoupled and functionally heterogeneous (Collins et al., 2002).

The ability of mitochondrial fusion to protect mitochondrial function is not limited to cultured cells. Using transgenic techniques, mice have been generated that contain varying ratios of mitochondria carrying mutant mtDNA and mitochondria carrying wild-type mtDNA (Nakada et al., 2001b). When tissues from such mice are stained for cytochrome oxidase (COX) activity (an activity dependent on both mtDNA and nuclear function), all the mitochondria in an individual cell are either homogeneously COX positive or COX negative. That is, any particular cell does not show
a mosaic of mitochondrial phenotypes. In fact, cells containing more than 60% mutant mtDNA still had fully functional, COX-positive mitochondria. These results suggest that mitochondria are not autonomous organelles, but rather cooperate to protect mitochondrial function even when wild-type mtDNA is in the minority.

V. Mitochondrial Dynamics and Human Disease

The results described above, indicating protective effects of mitochondrial cooperation, are relevant to understanding the pathogenesis of human diseases caused by mtDNA mutations (Larsson and Clayton, 1995; Wallace, 1999). It may explain why such diseases typically are not symptomatic until the burden of mutant mtDNA reaches a critical threshold. Furthermore, it may be possible to exploit this phenomenon to develop new therapies (Nakada et al., 2001a). Introduction of a modest amount of mitochondria carrying wild-type mtDNA may be sufficient to rescue the function of the entire mitochondrial population in mutant cells. Technically, this approach may be more feasible than gene therapy with mtDNA.

The importance of mitochondrial dynamics in human physiology is also clearly indicated by the discovery that the most common hereditary form of optic neuropathy, autosomal dominant optic atrophy (DOA) Kjer type, is due to mutations in \textit{OPA1} (Alexander et al., 2000; Delettre et al., 2000, 2002). Estimates of the prevalence of this disease range from 1 in 10,000 to 1 in 50,000. Although the disease has a variable presentation and penetrance, it typically presents in childhood with loss of visual acuity, visual field defects, and optic disc pallor. The pathophysiology appears to be a primary degeneration of retinal ganglion cells. The subsequent atrophy of the optic nerve gives rise to the pallor of the optic discs characteristic on fundoscopic examination. Affected patients in one family show heterogeneous clumping of mitochondria in monocytes (Delettre et al., 2000).

Large-scale sequencing of disease alleles carried out by several groups has uncovered dozens of molecular lesions in \textit{OPA1} leading to autosomal dominant atrophy (Delettre et al., 2002; Thiselton et al., 2002). From these studies, two types of mutations have been commonly found—missense mutations in the GTPase domain and truncation mutations located throughout the protein. There are at least two possible mechanisms through which \textit{OPA1} mutations may act. First, haploinsufficiency may be the cause of this dominant autosomal disease. Some of the severely truncated mutations probably act in this manner. Consistent with this model, one family with dominant optic atrophy has been reported to carry a deletion of one \textit{OPA1} allele (Marchbank et al., 2002). In another family, one compound heterozygous patient exhibited a much more severe disease phenotype than any
of her simple heterozygous family members (Pesch et al., 2001). Finally, some mutations truncate the protein so severely (including one that causes termination after just one residue) that mechanisms other than haploinsufficiency are unlikely (Delettre et al., 2002; Pesch et al., 2001). This genetic behavior, along with the OPA1 overexpression and underexpression studies described earlier, suggest that precise cellular levels of OPA1 are critical for maintaining normal mitochondrial morphology and function.

Although haploinsufficiency seems to account for some of the OPA1 mutations, the biochemical nature of OPA1 suggests an additional mechanism through which other mutations may act. Dynamin and dynamin-related proteins such as Drp1 are able to self-assemble (Shin et al., 1999; Smirnova et al., 2001; Yoon et al., 2001), a biochemical property that is essential for their role in membrane remodeling. As a result, GTPase mutants in dynamin and Drp1 often act as dominant negatives by incorporating into complexes with wild-type protein. It remains to be shown directly that OPA1 forms oligomers, but it would not be surprising if some OPA1 mutants similarly act in a dominant-negative manner by incorporating into wild-type complexes. Also, OPA1 mutants that are incorporated into larger fusion complexes, potentially including Mfn, may act as dominant negatives. There are two regions of OPA1 with coiled/coil motifs that may be involved in protein–protein interactions. Some disease alleles encode truncated OPA1 molecules that retain some of these candidate self-assembly domains. Further biochemical characterization of such alleles will be necessary to resolve whether any of them act in a dominant-negative manner.

Interestingly, a spontaneous semidominant mutation in the mouse, termed Bst (Belly Spot and Tail), maps close to the OPA1 locus and leads to optic nerve atrophy in heterozygous animals. These observations had raised the possibility that Bst may be a naturally occurring mouse model for human dominant autosomal optic atrophy (Rice et al., 1995). However, more detailed mapping and sequencing studies have established that Bst and OPA1 are actually two distinct loci (Delettre et al., 2003). Western analysis of Bst homozygous embryo lysates shows no loss of OPA1, and the mitochondria of mutant cells are morphologically indistinguishable from those of wild-type cells (Chen and Chan, unpublished results, 2003).

VI. Mitochondrial Dynamics in Vertebrate Development

Mitochondrial dynamics also appears to be important in tissues other than the retina. Early cytological and ultrastructural studies of mitochondria in vertebrate embryos suggest that mitochondrial morphology in specific tissues is developmentally regulated. For example, mitochondria in rat liver undergo a complex developmental pattern of morphological transitions. In
the early hepatic diverticulum of the embryonic rat, the mitochondria initially appear as minute spheres. Over the next several days, these mitochondria apparently coalesce into beaded filaments that later convert into smooth filaments shortly before birth (Smith, 1931). Although it is not possible to draw definitive mechanistic conclusions from these studies, it is reasonable to hypothesize that these morphological transitions result from upregulation of mitochondrial fusion or downregulation of fission during hepatocyte differentiation. Another striking example occurs during development of skeletal muscle in the rat diaphragm (Bakeeva et al., 1978, 1981). During embryonic stages, the muscle cells contain only a few mitochondria that form short tubules without interconnections. During the first two postnatal months, significant mitochondrial biogenesis occurs in association with a dramatic increase in interconnectivity of mitochondria. By 2 months, the muscle cells have developed a highly organized mitochondrial reticulum composed of interconnected mitochondrial tubules. It has been speculated that these long tubules may enable the transport of energy and substrates over long distances in muscle fibers (Bakeeva et al., 1978; Skulachev, 2001).

Studies of the mitofusins, Mfn1 and Mfn2, have provided clear evidence for the importance of mitochondrial fusion during mouse development. When either Mfn1 or Mfn2 is disrupted by homologous recombination techniques, midgestation embryonic lethality ensues (Chen et al., 2003). The cause of lethality in Mfn1-deficient mice is uncertain, but the loss of Mfn1 clearly affects development of the embryo proper. Mutant embryos are much smaller than their wild-type or heterozygous littermates and manifest developmental delay. The degree of delay is not uniform among various tissues, however, and therefore the embryos appear severely deformed (Chen and Chan, unpublished results, 2003). Great variation also exists between embryos, even among littermates.

In the case of Mfn2 disruption, the lethality has been traced to improper development of the placenta (Chen et al., 2003). Anatomically, Mfn2 mutant embryos are quite normal looking, albeit slightly smaller and delayed relative to their littermates. Remarkably, the extraembryonic defect is strikingly cell-type specific—of the three layers of the developing placenta, only the trophoblast giant cell layer shows a severe disruption in structure. The trophoblast giant cells are uniquely situated at the fetal–maternal boundary and are therefore critical for several processes essential for maintenance of the pregnancy, including invasion of the placenta into the maternal decidua, secretion of placental hormones, and development of the fetal–maternal vasculature (Cross, 2000). In trophoblast stem cell cultures, the Mfn2-deficient cells show spherical mitochondria, in contrast to the tubular mitochondria of wild-type cells. Interestingly, ultrastructural studies on preimplantation human embryos describe morphogenetic changes in mitochondrial structure during differentiation and hatching of the blastocyst (Sathananthan and Trounson, 2003).
The spherical or oval mitochondria of the oocyte and early embryo dramatically transform into long tubules with increased transverse cristae, an indication of increased metabolic activity. Trophoblast cells, in particular, contain extremely elongated mitochondria. The analysis of conditionally targeted alleles of *Mfn1* and *Mfn2* will allow the identification of additional developmentally regulated fusion events during vertebrate development.

### VII. Mitochondrial Dynamics and Apoptosis

Many lines of evidence implicate mitochondria as central regulators of programmed cell death. Under appropriate conditions, mitochondria trigger cell death by the release of apoptosis-promoting proteins sequestered in the intermembrane space (Desagher and Martinou, 2000; Newmeyer and Ferguson-Miller, 2003; Wang, 2001). For example, cytochrome *c*, Smac/DIABLO, and apoptosis-inducing factor (AIF) are released from the mitochondrial intermembrane space to promote later apoptotic events in the cytosol and nucleus.

Clearly, regulation of the permeability of the mitochondrial outer membrane is critical for precise control of cell survival. The mechanism of mitochondrial membrane permeabilization during induction of apoptosis is controversial and has been extensively discussed (Desagher and Martinou, 2000; Martinou and Green, 2001; Newmeyer and Ferguson-Miller, 2003; Wang, 2001; Zamzami and Kroemer, 2001, 2003). Two types of models have been proposed to explain mitochondrial membrane permeabilization. In the first scenario, members of the Bcl-2 family are thought to directly generate protein-conducting channels in the mitochondrial outer membrane. The Bcl-2 family of proteins can have either antiapoptotic (Bcl-2, Bcl-xL) or proapoptotic (Bid, Bax) functions. Although Bax normally resides in the cytosol, during induction of apoptosis it is activated by a cleaved form of Bid, undergoes a conformational change, and inserts into the mitochondrial outer membrane. It is thought that Bax may generate protein-conducting channels by forming large homotypic complexes. This idea has received support from structural studies revealing similarity between the structure of Bcl-2 family members and that of dipheria toxin and bacterial colicins, both pore-forming proteins (Muchmore et al., 1996; Suzuki et al., 2000). Furthermore, Bax and Bid can directly permeabilize vesicles *in vitro*, recapitulating some of the features of outer mitochondrial membrane permeabilization (Newmeyer and Ferguson-Miller, 2003; Zamzami and Kroemer, 2003). In this proposed type of membrane permeabilization, there is no immediate catastrophic damage to the mitochondria.

A second proposed mechanism for permeabilization of the mitochondrial outer membrane involves a phenomenon called the mitochondrial
permeability transition (Martinou and Green, 2001; Zamzami and Kroemer, 2001). In this model, a protein channel, called the permeability transition pore (PTP), opens and dissipates the inner membrane potential. The opening of the PTP leads to osmotic swelling of the mitochondrial matrix and consequently to rupture of the outer membrane, due to the much larger surface area of the highly involuted inner membrane surrounding the matrix. Rupture of the outer membrane allows release of apoptotic proteins trapped in the intermembrane space. At sites of contact between the outer and inner mitochondrial membranes, the PTP is composed of the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane, along with several other associated proteins. Bcl-2 family members may regulate apoptosis by modulating the opening of this channel. This mechanism would normally result in irreparable damage to the mitochondria, although modified versions of this model can accommodate transient or less severe damage.

Given the importance of mitochondria in many forms of apoptosis, does mitochondrial dynamics play a role in regulating this process? Specifically, do the pathways of mitochondrial fusion or fission intersect with those of apoptosis? These questions have been addressed only recently, and the evidence is intriguing (Karbowski and Youle, 2003). Some forms of programmed cell death are correlated with changes in mitochondrial morphology (Desagher and Martinou, 2000). For example, when COS-7 cells undergo apoptosis from overexpression of proapoptotic Bax or from treatment with compounds such as staurosporine or etoposide, they display concomitant fragmentation of the mitochondrial network into shorter tubules or spheres (Frank et al., 2001). During the fragmentation process, Drp1 relocates from a predominantly cytosolic pool to punctate spots on mitochondria. Overexpression of dominant-negative Drp1 in such cells prevents fragmentation and seems to suppress programmed cell death, as measured by mitochondrial membrane depolarization, cytochrome c release, and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining (Frank et al., 2001). Drp1 is also involved in the fragmentation of mitochondria observed during apoptosis induced by stimulation of death receptors (Breckenridge et al., 2003). In this case, activation of caspase 8 causes cleavage of BAP31, an integral membrane protein of the endoplasmic reticulum (ER). One of the BAP31 cleavage products, termed p20, induces Ca2+ release from the ER, followed by Drp1-dependent fragmentation of mitochondria. Expression of dominant-negative Drp1 prevents mitochondrial fragmentation and reduces both cytochrome c release and downstream caspase activation.

The possible connection of apoptosis with the mitochondrial fission pathway is also suggested by tracking the localization of Bax and Drp1 in apoptotic cells. Bax, normally a cytosolic protein, accumulates in punctate spots on mitochondria on induction of apoptosis (Karbowski et al., 2002).
These puncta of Bax localization colocalize with Drp1, thereby suggesting an association of an apoptotic stimulator with the fission machinery. The significance of these colocalizations, while intriguing, will need to be tested by functional studies.

hFis1, another component of the mitochondrial fission machinery, has also been implicated in apoptotic events. hFis1 overexpression leads to mitochondrial fragmentation (James et al., 2003; Yoon et al., 2003) and an increase in cytochrome c release and cell death (James et al., 2003). However, mitochondrial fission and cell death are not necessarily linked, because coexpression of dominant-negative Drp1 inhibited mitochondrial fragmentation (and cytochrome c release) without inhibiting cell death. Furthermore, Bcl-xL blocks apoptosis without affecting mitochondrial fragmentation.

If mitochondrial fission is involved in apoptosis, mitochondrial fusion may protect against cell death. Indeed, two components of the fusion pathway have been associated with apoptosis. Reduction of OPA1 levels by RNAi leads not only to fragmentation of the mitochondrial network but also to characteristics of programmed cell death (Olichon et al., 2003). Cells display loss of mitochondrial membrane potential, condensed chromatin, cleavage of poly(ADP-ribose) polymerase (PARP), and cytochrome c release. In addition, Mfn2 was found to colocalize with Bax and Drp1 during apoptosis (Karbowski et al., 2002). It is unclear what a fusion factor is doing at supposed sites of fission. This result is also perplexing, because other studies have shown that Mfn2 is localized uniformly to the mitochondrial membrane (Chen et al., 2003; Rojo et al., 2002; Santel and Fuller, 2001).

VIII. Perspectives and Future Challenges

Despite the identification of several important players in the control of mitochondrial dynamics, we still have little understanding of the molecular mechanisms through which mitochondria fuse and divide. The mechanisms will likely be novel, because unlike other well-studied membrane-remodeling events, such as vesicle fusion and viral entry, mitochondrial fusion and fission require the coordination of four separate bilayers. A structural understanding of the known proteins, along with extensive mutational analysis, will be required to gain a detailed model for their mode of action. In particular, it will be important to establish which proteins actually mediate the fusion/fission processes. Some of the known proteins physically interact to form complexes on the mitochondria. Further work will be required to elucidate whether these interactions serve to coordinate the inner and outer membranes. Although many tools are available for the study of mitochondrial dynamics, one serious gap has been the lack of a cell-free, biochemical assay for either fusion or fission. Such an assay would be invaluable for
dissecting the steps involved in both fusion and fission, as has been illustrated for the analysis of intracellular vesicular trafficking (Mellman and Warren, 2000; Wickner, 2002). Yeast genetics has identified many of the genes involved in regulating mitochondrial dynamics. Some of these yeast genes, such as \textit{DNM1} and \textit{FIS1}, have obvious mammalian homologs that have been shown to affect mammalian mitochondrial dynamics (James et al., 2003; Smirnova et al., 2001; Yoon et al., 2003). Other yeast genes, such as \textit{UGO1} and \textit{MDV1}, have no known homologs, and approaches other than bioinformatics will be required to identify their mammalian counterparts or other novel mammalian components.

To study the role of mitochondrial dynamics in vertebrate development, it is clear that development of animal models will be critical. In the case of the mitofusins, simple knockout alleles lead to midgestation embryonic lethality, precluding the ability to study later developmental effects and adult physiology (Chen et al., 2003). In such cases, it is clearly important to develop conditional alleles that allow temporal and spatial control of gene disruption. In the case of Mfn1s, such conditional alleles are being analyzed (Chen and Chan, unpublished results, 2003).

We are clearly in the beginning stages of understanding mitochondrial dynamics in mammals. It is becoming evident that this process is important in regulating mitochondrial morphology and function, and therefore is essential for embryonic development, but much more remains to be explored. In particular, the possible connections between mitochondrial dynamics and programmed cell death, aging, and mtDNA maintenance remain to be firmly established.

References


5. Mammalian Mitochondrial Dynamics


5. Mammalian Mitochondrial Dynamics


I. Introduction

Gene-specific repression of transcription plays a central role in gene regulation. Controlling gene activity is especially critical in development, during which boundaries of gene expression are often determined by the spatially restricted localization or activity of transcriptional repressors (reviewed in Mannervik et al., 1999). Repressors are also critical in the control of gene expression in response to extracellular signals. Genes are often maintained in an off state by a repressor protein until signal transduction alleviates the repression. Sequence-bound repressors are required to direct inhibition of transcription to specific genes at appropriate times, but these proteins cannot inhibit transcription on their own (reviewed in Maldonado et al., 1999; Roeder, 1998). Repressors collaborate with corepressor complexes that provide the molecular activities that mediate repression. Such complexes include...
N-CoR/SMRT, CtBP, Groucho, and Tup1–Ssn6 (Maldonado et al., 1999). These activities do not bind DNA directly but are brought to target promoters via interactions with sequence specific repressor proteins. It is not clear how these corepressors function to block transcription once recruited. However, many connections between corepressor functions and chromatin remodeling activities have been discovered, indicating that reorganization of chromatin is central to the repression process. For example, corepressor complexes such as N-CoR and SMRT harbor multiple histone deacetylase activities that are required for repression (reviewed in Jepsen and Rosenfeld, 2002). Changes in levels and patterns of histone methylation are also important in controlling gene expression and may directly influence the association of corepressor complexes with target promoters (reviewed in Zhang and Reinberg, 2001). Indeed, findings indicate that different patterns of histone modifications may constitute a code that is read by regulatory factors to achieve particular transcriptional states (Jenuwein and Allis, 2001). Here we review our current understanding of the functional interplay between corepressors and histone-modifying enzymes that likely play a role in establishing and reading such a code.

II. Histone Modifications and Regulatory Potential

DNA in eukaryotic cells is wrapped around an octamer of histone proteins, forming chromatin. The histone octamer contains two heterodimers of histones H2A and H2B and a tetramer of histones H3 and H4. Spooling of 146 base pairs of DNA around the histone octamer completes the nucleosome, the basic repeat unit of chromatin (reviewed in Wolffe, 1998).

Each of the core histone proteins is composed of a globular central region and a flexible N-terminal region that extends from the nucleosome core particle. Although they are not required for maintenance of nucleosome structure, the N-terminal regions of the histones are highly conserved, and contain several residues that are subject to a variety of posttranslational modifications. These modifications include acetylation, phosphorylation, methylation, and ubiquitination. Histone modifications play a regulatory role in gene expression by altering higher order chromatin structures and by providing docking sites for nonhistone regulatory proteins (reviewed in Jenuwein and Allis, 2001; Strahl and Allis, 2000). The last decade has brought a wealth of information in both identifying the enzymes responsible for regulating these modifications and for understanding the molecular functions of the modifications.
A. Histone Acetylation

Acetylation and deacetylation of conserved lysine residues in the amino-terminal histone tail domains are highly regulated events that play important roles in transcriptional regulation. Transcriptionally silent genes tend to be associated with hypoacetylated histones, whereas transcriptionally active genes tend to be associated with hyperacetylated histones (reviewed in Fischle et al., 2003; Struhl, 1998; Wolffe, 2001). Acetylation is catalyzed by histone acetyltransferases (HATs), and the acetyl groups are removed by histone deacetylases (HDACs). Acetylation affects the electrostatic charge of the histones, which may loosen some DNA–histone contacts to facilitate transcription. Acetylation also affects nucleosome–nucleosome interactions in vitro, leading to the unfolding of higher order chromatin packing (reviewed in Hansen, 2002; Hayes and Hansen, 2001). Deacetylation of the histones reverses these effects. In addition, acetylation and deacetylation directly influence the association of other proteins with the histones. Several corepressors, such as Tup1 and the related Groucho/TLE proteins, bind well to hypoacetylated histone tails but do not bind to hyperacetylated histones. Conversely, acetylated histones provide a docking site for bromodomain-containing proteins, such as Gcn5 (a HAT) or Swi2 (an ATP-dependent chromatin-remodeling activity), which are often associated with transcriptional activation (Hassan et al., 2002). Histone acetylation and deacetylation, then, is thought to provide a rapid trigger for changes between active and inactive transcription states.

B. Histone Methylation

Changes in histone methylation are also associated with changes in transcription states. However, unlike acetylation, methylation may not be readily reversible as no demethylase activities have been identified. Histone methylation may not trigger changes in transcription states but may rather provide a “memory” function, distinguishing regions that have never been transcribed from those that have been active at some point in the life of the cell (Ng et al., 2003b; Turner, 2003). The methylation of histones can take several forms. Lysine and arginine residues can be mono-, di-, or trimethylated. Each level and site of methylation appears to have distinct functions. For example, trimethylated lysine 4 (K4) in H3 is associated with active genes in Saccharomyces cerevisiae, whereas the dimethyl form of K4 is associated with both repressed and active genes (Santos-Rosa et al., 2002). In contrast, methylation of K9 in H3 is often associated with
gene silencing and heterochromatin formation (reviewed in Bannister et al., 2002; Kouzarides, 2002; Zhang and Reinberg, 2001).

Lysine methylation can affect the interactions of regulatory proteins with chromatin in at least three ways. First, methylation can provide a docking site for chromodomain-containing proteins. Second, it can block the interactions of regulatory factors with particular nucleosomes. Finally, methylation at one site can influence the occurrence of other histone modifications in the same histone tail. The first example of chromodomain docking was the observation that methylation of K9 in H3 by the Su(var)3-9 methyltransferase provides a specific binding site for heterochromatin protein 1 (HP1) (Bannister et al., 2001). Methylation of H3 K27 by the E(Z) protein, a subunit of the ESC–E(Z) complex, facilitates repression and stable association of polycomb group proteins with specific target loci (Cao et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002; Plath et al., 2003). These docking events are quite specific. HP1 does not bind methylated K27 and polycomb does not bind methylated K9. Neither HP1 nor polycomb binds to methylated K4 in H3. Methylation of certain lysines can also block binding of regulatory proteins. The corepressor complex NuRD is repelled by H3 K4 methylation (Zegerman et al., 2002). This effect is again specific, as H3 K9 methylation does not prevent binding. H3 K4 methylation is found at sites of active transcription, consistent with a role in disrupting the association of histones with repressor complexes. Histone H3 K79 methylation by the Dot1 protein in S. cerevisiae also blocks binding of regulatory proteins. H3 K79 methylation is required for efficient silencing at HM loci and telomeres, but this modification is not enriched at these sites. Rather, K79 methylation blocks the binding of silencing factors (Sir proteins) at other regions of the genome. When this methylation event is lost, titration of these factors away from silent sites leads to a loss of silencing (Ng et al., 2003a; van Leeuwen et al., 2002). Histone H3 K79 methylation is highly conserved, and a human Dot1-like protein, DOT1L, also houses H3 K79 methyltransferase activity (Feng et al., 2002). Interestingly, methylation at one site modulates other modifications in the same histone tail. For example, the methylation of H3 at K9 inhibits phosphorylation at S10 (Rea et al., 2000). Because S10 phosphorylation can enhance acetylation of K14 in H3 (Cheung et al., 2000; Lo et al., 2000) but inhibits acetylation of K9 (Edmondson et al., 2002), methylation of K9 could have many downstream consequences that ultimately affect cofactor binding and determine transcriptional outcomes. Transregulation of histone modifications has also been observed. Ubiquitination of K123 in H2B is required for H3 K4 and K79 methylation but not for K36 methylation (Briggs et al., 2002; Sun and Allis, 2002).
III. Corepressors and Histone-Modifying Activities

As seen above, histone modifications can profoundly influence the association of regulatory activities with chromatin (Fig. 1). Not surprisingly, then, many corepressor complexes contain or recruit histone-modifying activities. A few examples of such interactions are described here to illustrate the fundamental connections between transcriptional repression and the organization of chromatin.

A. Tup1–Ssn6 and Groucho/TLE

The Tup1–Ssn6 corepressor regulates up to 3% of the *S. cerevisiae* genome (DeRisi et al., 1997). Tup1–Ssn6 is highly conserved within yeast, with homologs in *Schizosaccharomyces pombe* and *Candida albicans*. Although less well conserved in higher eukaryotes, every eukaryotic organism studied contains repressors that resemble Tup1 and Ssn6 in sequence and in function. Tup1 contains a WD repeat motif that is similar in sequence and organization to WD domains present in the *Drosophila* Groucho (Gro) protein.

Figure 1  Summary of interactions between regulatory factors and modified histone H3. (A) Acetylation of H3 (and H4, not shown) promotes binding of bromodomain proteins, including Swi2 (the ATPase subunit of the Swi/Snf chromatin-remodeling complex) and TAF1 (largest subunit of metazoan TFIID, also known as TAFI1250). Hypoacetylated histones can interact with the corepressors Tup1–Ssn6 and the Groucho/TLE family. (B) Methylation and phosphorylation sites affect regulatory protein binding and other modifications. Methylation can promote (green arrows) or inhibit (red bars) the binding of regulatory factors. Phosphorylation and methylation can also inhibit additional posttranslational modifications (black bars).
mammalian Transducin-like Enhancer of Split (TLE) proteins, and TBL1-TBLR1 family members (reviewed in Courey and Jia, 2001). Like Tup1, Groucho is a corepressor of many diverse genes. Groucho can be recruited to target genes by many transcription factors including the Hairy- and Runt-related proteins, Dorsal, Engrailed, and TCF. TLE proteins are recruited by AML proteins and are important for hematopoiesis and osteogenesis (reviewed in Chen and Courey, 2000). In addition, TLE1 interacts with LEF1 and is important in repressing β-catenin-regulated genes in the absence of Wnt signals (Levanon et al., 1998). At least two of these proteins, TLE1 and TLE2, interact with proteins related to Ssn6, furthering the analogies between these corepressors (Grbavec et al., 1999).

Like Tup1, the TLE proteins and Groucho associate with underacetylated histone tails (Palaparti et al., 1997). As described below, Tup1–Ssn6 can interact with multiple histone deacetylases, including Rpd3 (Watson et al., 2000; Wu et al., 2001). Similarly, Groucho interacts with Rpd3 (Flores-Saaib and Courey, 2000) and TLE–AML3 complexes interact with HDAC6 (Westendorf et al., 2002). For both Tup1–Ssn6 and Groucho/TLE, histone deacetylase activity is required for efficient repression in vivo (Chen et al., 1999; Watson et al., 2000).

B. SMRT/N-CoR

The nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) are two highly related corepressors that mediate repression by unliganded nuclear hormone receptors such as the thyroid hormone receptor and the retinoic hormone receptor (reviewed in Jepsen and Rosenfeld, 2002). N-CoR and SMRT are similar in sequence, but are not completely redundant in function (Jepsen et al., 2000). Depending on the purification strategy, these proteins can be found in several distinct macromolecular complexes. These complexes always include WD repeat proteins similar to Tup1. SMRT is found in a complex with TBL1 (Guenther et al., 2000; Li et al., 2000) and N-CoR is found in a complex with both TBL1 and TBLR1 (Yoon et al., 2003). Like Tup1, the TBL proteins interact with histones (Yoon et al., 2003).

N-CoR and SMRT can interact with several different histone deacetylases. There are two distinct classes of histone deacetylases based on homologies to the yeast HDACs. Class I HDACs (HDAC1–3 and 8) are homologous the yeast HDAC Rpd3, and class II HDACs (HDAC4–7) are homologous with the yeast Hda1 protein. N-CoR and SMRT primarily associate with TBL proteins and HDAC3, a class I HDAC (Guenther et al., 2000; Li et al., 2000; Wen et al., 2000). Class II HDAC4, -5, and -7 can interact with N-CoR and SMRT in vitro (Huang et al., 2000; Kao et al., 2000). However, as shown for HDAC4
and -5, the class II proteins have no enzymatic activity when in complexes with N-CoR and SMRT. The class I HDAC3 provides all the enzymatic activity of the complex (Fischle et al., 2002). Thus, it is suggested that the class II HDACs might act to bridge the enzymatically active SMRT/N-CoR–HDAC3 complex to select transcription factors. SMRT and N-CoR can also associate with an additional corepressor complex, mSin3A (Heinzel et al., 1997; Nagy et al., 1997).

C. Sin3

The Sin3 corepressor is highly conserved between yeast and higher eukaryotes. In *S. cerevisiae*, Sin3 associates with the class I HDAC Rpd3 and is recruited to target genes by DNA-bound repressors such as Ume6. As with Tup1–Ssn6, this leads to a localized deacetylation of histones at promoter regions of target genes (Kadosh and Struhl, 1998; Rundlett et al., 1998). The mammalian orthologs, mSin3A and mSin3B, are found in large complexes with additional associated proteins, including class I HDAC1 and -2 (reviewed in Ahringer, 2000; Knoepfler and Eisenman, 1999; Ng and Bird, 2000).

D. CtBP

The transcriptional corepressor CtBP (C-terminal binding protein of E1A) plays important roles both in development and in oncogenesis (reviewed in Chinnadurai, 2002). CtBP is recruited to target genes through interactions with a PLDLS motif in transcriptional repressors such as the E1A family. It can mediate repression through histone deacetylase-dependent or -independent mechanisms, depending on the promoter context. Interestingly, CtBP is found in a multisubunit complex that contains both histone deacetylase (HDAC1 and -2) and histone methyltransferase activities (G9A and Eu-HMTase1) (Shi et al., 2003). This complex can deacetylate H3 K9 and subsequently methylate H3 K9. Intriguingly, the CtBP complex also contains chromodomain proteins (HPC2 and CDYL). It will be interesting to see whether this complex also docks onto methylated K9 through these chromodomain proteins to create a self-reinforcing repressive state.

E. Rb

The retinoblastoma (Rb) protein family members also recruit histone modifiers. Rb proteins associate with the E2F family of DNA-binding transcription factors, sequestering E2F as well as actively repressing E2F target
genes. HDAC1 and the histone methyltransferase Suv39H1 interact directly with the Rb family of pocket proteins, and both histone deacetylation and histone methylation contribute to Rb-mediated repression (Brehm et al., 1998; Luo et al., 1998; Vandel et al., 2001). Suv39H1 methylates H3 K9, facilitating the binding of HP1, and HP1 also functions in Rb-mediated repression (Nielsen et al., 2001). Interestingly, the cell cycle-dependent phosphorylation of Rb appears to release HDAC1 from its binding site in the pocket domain, thereby relieving active repression by E2F (Harbour et al., 1999).

F. NuRD

As its name implies, the corepressor NuRD (nucleosome remodeling and deacetylase) has two important connections to chromatin. The multisubunit complex contains both ATP-dependent chromatin-remodeling activity and histone deacetylase activity (reviewed in Knoepfler and Eisenman, 1999; Ng and Bird, 2000). The chromatin-remodeling activity is contained in the chromodomain protein Mi-2. RNA interference (RNAi) experiments in Caenorhabditis elegans have shown that NuRD complex members are required for embryonic patterning, Hox expression and function, Wnt signaling, and antagonizing the Ras pathway (Ch’ng and Kenyon, 1999; Herman et al., 1999; Solari et al., 1999). NuRD and Sin3 are the two major histone deacetylase complexes in the cell, and they share several associated polypeptides (reviewed in Ahringer, 2000). HDAC1 and HDAC2, both class I histone deacetylases, are found in both the NuRD and Sin3 complexes. In addition to the chromatin modifying activities of NuRD, NuRD cannot bind in vitro to H3 when K4 is methylated (Zegerman et al., 2002). NuRD binds well to an unmodified H3 tail, and methylation of K9 (associated with repression) does not affect binding. Thus K4 H3 methylation likely excludes the corepressor NuRD from active genes.

G. Summary

Every corepressor described above has a least one connection to chromatin remodeling, and these are just a few examples of the connections described in the current literature. Despite these many examples, however, it is still not clear how chromatin-modifying activities are coordinated with other functions within corepressor complexes, or how these activities actually affect the process of transcription. Study of the Tup1–Ssn6 corepressor in the genetically tractable yeast S. cerevisiae has offered some unique insights into these questions.
IV. Tup1–Ssn6 Corepressor Complex: A Model for Corepressor Flexibility?

Tup1–Ssn6 mediates repression of a large and diverse set of genes in *S. cerevisiae* (reviewed in Smith and Johnson, 2000). Examples of gene classes regulated by this corepressor complex are genes that are repressed by glucose (e.g., *SUC2*), genes that respond to hypoxia (e.g., *ANB1*), genes induced by DNA damage (e.g., *RNR2*) and cell type-specific genes (e.g., *STE6*). In many cases, the specific DNA-binding partner that recruits the corepressor is known. For example, Tup1–Ssn6 interacts with α2/Mcm1 (cell type-specific genes), Crt1 (DNA damage-inducible genes), and Rox1 (oxygen utilization genes). Thus, the exact point of Tup1–Ssn6 recruitment to a number of targets is well defined, facilitating assessment of the molecular consequences of corepressor binding.

Both Tup1 and Ssn6 contain repeat motifs that may give them a somewhat flexible structure. As mentioned above, Tup1 contains a WD motif, named for the characteristic placement of tryptophan (W) and aspartate (D) with a 40 amino acid domain. The WD motifs in a number of proteins fold into seven bladed propeller structures that form an overall donut shape and provide a protein-protein interaction interface (Sprague *et al.*, 2000). Ssn6, also called Cyc8, contains a tetratricopeptide repeat (TPR), another protein–protein interaction module (Blatch and Lassle, 1999). The Tup1–Ssn6 complex is composed of four copies of Tup1, and one copy of Ssn6 (Redd *et al.*, 1997; Varanasi *et al.*, 1996).

The molecular mechanism by which Tup1–Ssn6 functions is not fully understood, but Tup1–Ssn6 likely uses both interactions with chromatin and interactions with the general transcription machinery to achieve repression. Many subunits of the mediator complex that is associated with the CTD (C-terminal domain) of the largest subunit of RNA polymerase II interact both genetically and physically with Tup1–Ssn6. Mutations in several genes encoding mediator components including Sin4, Srb10/11, Med3, and Srb7 result in partial derepression of Tup1–Ssn6-regulated genes (reviewed in Carlson, 1997). Physical interactions have been detected between Tup1 and Srb7 (Gromoller and Lehming, 2000), Med3 (Papamichos-Chronakis *et al.*, 2000), and Srb10/11 (Schuller and Lehming, 2003; Zaman *et al.*, 2001). Tup1 also interacts with Cet1, the mRNA 5’-triphosphatase that is a subunit of the RNA-capping enzyme associated with the CTD. As CET1 is essential, effects of CET1 deletion on the repression of Tup1–Ssn6-regulated genes could not be examined, but overexpression of Cet1 compromises the repression of a-cell specific reporter (Mukai *et al.*, 2003). Collectively, these observations indicate that Tup1–Ssn6 may directly
influence, or is influenced by, multiple proteins that are directly bound to the CTD. The consequences of these interactions, however, are still unknown.

Tup1–Ssn6 also directly interacts with histones and influences the organization of chromatin. Certain repressed genes under Tup1–Ssn6 control are packaged into highly positioned nucleosomes during repression (Cooper et al., 1994; Fleming and Pennings, 2001; Li and Reese, 2001; Roth et al., 1990). Although positioned nucleosomes are not present at every Tup1–Ssn6-regulated gene, histones H3 and H4 contribute to repression of all Tup1–Ssn6 targets, supporting a general role for chromatin in the repression mechanism. Tup1 binds preferentially to underacetylated H3 and H4 amino-terminal histone tails \textit{in vitro}, and combined mutation of the H3 and H4 tails leads to a large derepression of Tup1–Ssn6-regulated genes \textit{in vivo} (Edmondson et al., 1996). Chromatin immunoprecipitation experiments indicate that Tup1 binding \textit{in vivo} is associated with decreased acetylation of H3 and H4 (Bone and Roth, 2001; Davie et al., 2002; Deckert and Struhl, 2001). Accordingly, histone deacetylase activities are required for Tup1–Ssn6 repression (Watson et al., 2000; Wu et al., 2001). Combined loss of three class I histone deacetylases, Rpd3, Hos1, and Hos2, completely abolishes Tup1–Ssn6 repression at all genes examined (Watson et al., 2000). The class II deacetylase, Hda1, shows partial derepression of \textit{ENA1}, another Tup1–Ssn6-regulated gene (Wu et al., 2001). Physical interactions can be observed between each of these deacetylases (Rpd3, Hos1, Hos2, and Hda1) and the Tup1–Ssn6 complex (Davie et al., 2003; Watson et al., 2000; Wu et al., 2001).

At present, the role of histone methylation, if any, in Tup1–Ssn6-mediated repression is unknown. \textit{Saccharomyces cerevisiae} lacks an Su(var)3–9 homolog as well as detectable H3 K9 methylation that is associated with gene silencing in other organisms. However, \textit{S. cerevisiae} contains seven SET domain-containing proteins, and two of these, Set1 and Set2, have demonstrated histone methyltransferase activity. Set1 mediates H3 K4 methylation, as described above (Briggs et al., 2001). K4 trimethylation is correlated with both repression and activation (Santos-Rosa et al., 2002). No role for Set1 or K4 methylation in Tup1–Ssn6 repression has yet been reported. H3 K36 is methylated by the Set2 methyltransferase, and this event has also been associated with repression when Set2 is artificially tethered to a reporter promoter by fusion to the LexA DNA-binding domain (Strahl et al., 2002). It is not known whether H3 K36 methylation or Set2 normally functions in repression. Intriguingly, both Set1 and Set2 associate with specific modified forms of the C-terminal domain (CTD) of RNAPII. Set1 associates with the Ser5 phospho forms of the CTD that is associated with the switch from transcription initiation to elongation (Ng et al., 2003b). Set2 associates with the Ser2 phospho form of the CTD that is associated with transcription elongation (Li et al., 2002, 2003; Xiao et al., 2003). It is completely unknown
how or whether these associations are related to repression by Tup1–Ssn6. It will be interesting to see whether Tup1–Ssn6 utilizes any interactions with methyltransferases or the methylated histone tails to mediate repression of target promoters.

Taken together, the data in hand suggest a self-reinforcing model for Tup1–Ssn6 repression that involves interactions with specific repressor proteins and histone deacetylation. The Tup1–Ssn6 complex recruits histone deacetylases when it itself is recruited to target promoters by DNA-bound repressor proteins. This HDAC recruitment leads to a localized deacetylation of histones near the Tup1–Ssn6 recruitment site. Tup1 can then bind to these underacetylated tails, forming a stable repressive structure. If the interactions between Tup1 and the histones are disrupted, then the corepressor cannot be stably retained at target promoters, leading to a loss of repression. One outcome of Tup1–Ssn6 recruitment is a blockage of TBP binding to target promoters (Kuras and Struhl, 1999). This interference likely reflects a central step in the repression mechanism that might result from either Tup1–Ssn6 interactions with mediator components or the organization of a repressive chromatin architecture. These two aspects of the process of repression are likely related, but the kinetics of repression are presently unknown. Interactions between Tup1–Ssn6 and mediator components may be important for steps upstream or downstream of chromatin organization. For example, Tup1–Ssn6 might form a stable repressive chromatin architecture and then sequester mediator subunits to prevent the reinitiation of transcription. Alternatively, interactions between the corepressor and mediator components might be necessary to halt transcription and provide a window of opportunity for chromatin remodeling. Such kinetic considerations are relevant to any corepressor complex as they are fundamental to understanding the process of transcriptional repression.

The flexibility of the Tup1 and Ssn6 proteins may allow them to create gene-specific chromatin architectures. Such architectures may reflect different patterns of Tup1 association with different target genes. For example, chromatin immunoprecipitation experiments indicate that Tup1 is localized differently at STE6, a cell type-specific gene, and RNR2, a DNA damage-inducible gene (Davie et al., 2002). At STE6, Tup1 is recruited to the α2/Mcm1 repressor-binding site, but Tup1 association extends in a unidirectional manner from this site into the first several hundred base pairs of the STE6 coding region. In contrast, Tup1 is localized only to the Crt1 recruitment site at RNR2. In both cases, histone deacetylation tracks Tup1 locations, again indicating that deacetylation is important for repression but that different domains of deacetylation are established at different promoters. The possibility that Tup1–Ssn6 forms alternate structures at different target promoters was first proposed on the basis of data indicating that different domains of Tup1 and Ssn6 are required for repression of different
target genes (Smith and Johnson, 2000). Interactions of different WD domains in Tup1 or different TPR domains in Ssn6 with different DNA-bound repressor proteins might confer different conformations on the Tup1–Ssn6 complex. Such flexibility was proposed to allow Tup1–Ssn6 to accommodate differences in the spacing between repressor-binding sites and transcription start sites among different classes of target genes. Different corepressor conformations might also facilitate interactions with different HDAC activities, allowing gene-specific tailoring of deacetylation events. Because many corepressors interact with multiple HDACs, it will be especially interesting to determine whether such flexibility is a fundamental characteristic of corepressor functions.

The flexibility of Tup1–Ssn6 might even allow it to switch from a corepressor to a coactivator. At most genes studied to date, Tup1 is enriched at target promoters when those promoters are repressed (Davie et al., 2002; Ducker and Simpson, 2000; Wu et al., 2001). However, on GAL1 or GRE2, Tup1 can be detected during both repression and activation (Papamichos-Chronakis et al., 2002; Proft and Struhl, 2002). In addition, Tup1 is necessary for the recruitment of the SAGA histone acetyltransferase complex and the Swi/Snf chromatin-remodeling complex during activation. Surprisingly, in the absence of Tup1, SAGA and Swi/Snf are not required for activation (Proft and Struhl, 2002). These data might indicate that both chromatin modification and chromatin remodeling are utilized by Tup1–Ssn6 in both establishing and relieving repression. It appears that Tup1 can be flexible in the way it interacts with target promoters, and it does so in a gene-specific manner. Taken together, these data suggest that the Tup1–Ssn6 complex is a dynamic moiety that uses multiple interactions and multiple mechanisms to control gene expression.

There is still much to understand about how Tup1–Ssn6 utilizes interactions with chromatin to mediate repression at all the genes it regulates. Interactions with four histone deacetylases in Saccharomyces cerevisiae have been reported with this corepressor complex. Are all four HDACs used by the corepressor at every promoter or are specific deacetylases used at different classes of gene targets? Is histone deacetylation on Tup1–Ssn6 recruitment a preamble to specific histone methylation events? Does Tup1–Ssn6 utilize different combinations of deacetylase and methyltransferase activities to establish gene-specific histone codes? If so, how are these codes used to create gene-specific repressive chromatin architectures? How do the mediator subunits contribute to the mechanism of repression? These questions are pertinent to the functions of all corepressors. Future studies of the Tup1–Ssn6 complex will no doubt continue to provide insights concerning the flexibility and specificity of corepressor modes of action.
V. Summary and Future Questions

What we have learned about Tup1 has greatly aided our understanding of other corepressors in higher eukaryotes. As discussed above, the Groucho/TLE family is highly related to Tup1–Ssn6 and shares many functional similarities with Tup1–Ssn6. An intriguing parallel to Tup1–Ssn6 are the N-CoR and SMRT corepressors. They share many interesting similarities with Tup1–Ssn6, but the most striking similarity is that they, like Tup1–Ssn6, interact with both class I and class II histone deacetylases (Fig. 2). Whereas N-CoR and SMRT interact with both class I HDAC3 and the class II HDAC4, only class I HDAC3 has enzymatic activity. Both Tup1–Ssn6 and N-CoR/SMRT utilize distinct domains to interact with different histone deacetylases. It will be interesting to determine how Tup1–Ssn6 uses the class I and class II activities, and whether enzymatic HDAC activity is required for both classes. N-CoR and SMRT are found in multiple complexes with different partners, suggesting that these proteins are acting as docking platforms for regulatory information. It will be interesting to see how related the ultimate mechanisms of repression are between these highly related proteins.

**Figure 2** Tup1–Ssn6 shares many functional similarities with SMRT/N-CoR. SMRT and N-CoR are in complexes with TBL proteins that are highly related to Tup1. Both complexes can interact with both class I and class II HDACs.
Although the molecular mechanism of corepressor function is still unknown, many corepressor complexes can either modify or interact with chromatin. Thus, investigating the effects on chromatin is central to understanding how corepressors mediate repression. The histone code hypothesis suggests that the modified histone tails provide docking sites for regulatory proteins and many examples of regulatory proteins docking on modified histone tails have emerged. In a broader sense, the fundamental concept that a transcription regulatory protein can reinforce its stable interaction with target promoters, both by binding to specific modified forms of histones and by recruiting the activity needed to create that modified state, may be broadly applicable. Interactions between regulatory proteins, histones, and histone-modifying enzymes are a fundamental and conserved mechanism governing gene expression.

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6. Histone Modifications in Corepressor Functions


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I. Introduction

Abl is a ubiquitous nonreceptor tyrosine kinase involved in signal transduction. Genetic ablation of Abl in mice results in embryonic and neonatal lethality accompanied by a range of low-penetrant phenotypes, including reduced fertility, forshortened crania, defective lymphocyte maturation, and dysfunctional osteoblasts (Li et al., 2000; Schwartzberg et al., 1991; Tybulewicz et al., 1991). The pleiotropy of Abl-deficient phenotypes is suggestive of developmental flaws not amenable to detection by currently available histological examinations. The available information about Abl cellular function has yet to provide a coherent explanation of these pleiotropic defects in Abl-deficient mice.

Like all signal transducers, Abl effectuates its biological effects through interactions with other cellular components. These interactions depend on
multiple functional domains of Abl (Fig. 1). At the N terminus of Abl is an SH3 domain, which binds peptides with the PXXP motif. A short linker connects the SH3 domain to an SH2 domain, which binds peptides containing the Y(p)XXP motif. The SH2 domain is connected through another linker to the tyrosine kinase domain. The N-terminal SH3–SH2–kinase domain of Abl is homologous to the SH3–SH2–kinase domain of the Src family nonreceptor tyrosine kinases, both in primary sequence and in three-dimensional structure. Like Src family members, the SH3 and SH2 domains of Abl engage in intramolecular interactions with the kinase domain to form an autoinhibited conformation (Harrison, 2003; Nagar et al., 2003). Disruption of the autoinhibited conformation would lead to Abl kinase activation and freeing of the SH3 and SH2 domains to recruit substrates or other interacting proteins.

Several other domains in the large C-terminal region of Abl further regulate the signaling function of this tyrosine kinase. Most importantly, the C-terminal region directs Abl to different subcellular localizations. Abl contains three nuclear localization sequences (NLS) interdispersed with several proline-rich motifs that bind SH3 domains of other proteins and three HMG-like boxes (HLBs) that bind DNA. In the nucleus, Abl is localized to the chromatin through its interaction with DNA and other nuclear proteins (see below). At the C terminus of Abl is an actin-binding domain.

Figure 1 Schematics of the functional domains in Abl. (A) A linear diagram of Abl. Abl(1b) is myristoylated at the N terminus. Abl(1a) arises from a different promoter in the Abl gene, and lacks the N-terminal myristoylation. SH3, Src homology 3 domain; SH2, Src homology 2 domain; NLS, nuclear localization signal; NES, nuclear export signal. Proline-rich motifs are interposed among the three NLS and they bind to SH3 domains of Abl-interacting proteins. Abl binds A/T-rich DNA sequence and three HMG-like boxes mediate this binding in a cooperative manner. Abl binds G-actin and F-actin, and these binding functions are contained within a C-terminal 165-aa fragment. The extreme C terminus of Abl shares limited sequence homology with the calponin homology domain found in a variety of F-actin-binding proteins (Gimona et al., 2002; McWhirter et al., 1993; Sutherland-Smith et al., 2003). The NES is embedded within the F-actin-binding domain. Three caspase cleavage sites are denoted with arrows. Abl can become phosphorylated on two tyrosines in the kinase domain. Y245 is in the linker region connecting SH2 domain with the kinase domain. Y412 is in the activation loop of the kinase domain. Phosphorylation at either of these two sites may disrupt the autoinhibited conformation of Abl. (B) Two-dimensional representation of the autoinhibited conformation of Abl. Intramolecular interactions between the SH3, SH2, and kinase domains mediate the assembly of an autoinhibited conformation of Abl. In this inhibited conformation, the SH3 domain contacts the SH2 kinase linker, which in turn contacts the ATP-binding N lobe of the kinase domain. The Abl inhibitor, STI571, binds to the N lobe. The SH2 domain contacts the C lobe of the kinase domain and this contact requires bending of Cα-helix 1 in the C lobe. Interestingly, bending of this helix requires myristate binding to the C lobe. Thus, the N-terminal myristoyl group of Abl(1b) participates in establishing the autoinhibited conformation (Nagar et al., 2003). The relative positioning between the autoinhibited Abl kinase and the C-terminal region is unknown at this time. As discussed in text, Abl NLS is likely to be regulated by the kinase conformation. Only the inactive Abl is imported into the nucleus.

7. Death by Abl: A Matter of Location
that interacts with G- and F-actin. A nuclear export sequence (NES) is embedded in the actin-binding domain. In the cytoplasm, Abl interacts with the actin cytoskeleton (Woodring et al., 2000). The NLS and NES enable Abl to course in and out of the nucleus (Taagepera et al., 1998). Therefore, the Abl tyrosine kinase joins a growing number of signal transducers that undergo the process of nucleocytoplasmic shuttling.

Nucleocytoplasmic shuttling has emerged as an important mechanism to control the access of signaling molecules to the nucleus (Komeili and O’Shea, 2000). Several families of transcription factors, including NFAT, NF-κB, STAT, and others, are kept latent in the cytoplasm. In response to the appropriate signals, these transcription factors are imported into the nucleus to alter gene expression and then exported back to the cytoplasm on signal termination. For instance, cytoplasmic NFAT responds to calcium influx with a rapid translocalization into the nucleus; it is then exported out of the nucleus as the intracellular calcium level subsides (Graef et al., 2001; Zhu and McKeon, 1999). On the other hand, p53 is coexported out of the nucleus with Mdm2 to be destroyed by polyubiquitination and proteosome-mediated degradation (Geyer et al., 2000; Michael and Oren, 2003; Middeler et al., 1997; Tao and Levine, 1999). DNA damage signal disrupts the p53–Mdm2 interaction, retaining p53 in the nucleus to activate transcription (Michael and Oren, 2003). The nucleocytoplasmic shuttling of Abl suggests its biological functions are influenced by its subcellular location. This chapter examines the literature for regulation of Abl subcellular location and for location-dependent functions of Abl tyrosine kinase.

II. Nucleocytoplasmic Shuttling of Abl

A. Abl NLS and NES

1. NLS

Abl contains three NLS, each of which can independently direct Abl into the nucleus. NLS-1 is a bipartite NLS located immediately C terminal of the kinase domain (Fig. 1). NLS-2 and NLS-3 are not bipartite, and are embedded in the DNA-binding domain (Fig. 1). Simultaneous mutation of all three NLS is necessary to annul the nuclear import of Abl (Taagepera et al., 1998; Wen et al., 1996). Notwithstanding its three NLS, Abl is not exclusively nuclear. In cultured fibroblasts, Abl is evenly distributed in the cytoplasm and the nucleus.
2. NES

That Abl undergoes nuclear export is suggested by its nuclear accumulation in cells treated with leptomycin B (LMB) (Taagepera et al., 1998; Vella et al., 2003; Vigneri and Wang, 2001), which alkylates and inactivates exportin 1 (CRM1) (Kudo et al., 1998, 1999). Exportin 1 binds to nuclear export signal (NES) with a leucine-rich sequence motif (Fornerod et al., 1997; Stade et al., 1997). The Abl NES was identified by virtue of its ability to substitute for the NES of HIV Rev protein to drive its nuclear export (Taagepera et al., 1998). The Abl NES is stronger than many NES in its ability to drive the export of a reporter protein. It slightly trails the NES of PKI and MAPKK and ties with the NES of Rev, but surpasses the NES of Ran-BP1, FMRP, I-κB, and Mdm2 (Henderson and Eleftheriou, 2000). Mutation of a single leucine in the Abl NES causes the nuclear accumulation of Abl (Taagepera et al., 1998). Therefore, the subcellular distribution of Abl is determined by a dynamic equilibrium between its nuclear import and export.

3. Abl NES Is Stronger Than the Three NLS

To examine the balance of Abl NLS and NES function, we have fused green fluorescent protein (GFP) with the three Abl NLS in two formats, either with or without the Abl NES (Fig. 2A). The three Abl NES drove GFP into the nucleus, with no detectable cytoplasmic localization of GFP. Appending Abl NES to the GFP–Abl–NLS fusion resulted in a predominantly cytoplasmic distribution (Fig. 2B), showing Abl NES to outperform Abl NLS. Therefore, when the NLS and NES of Abl are separated from the rest of the Abl protein, the equilibrium favors a cytoplasmic location of Abl. This intrinsic equilibrium must be augmented in the context of Abl protein to achieve the even distribution of Abl between the cytoplasmic and nuclear compartments.

B. Regulation of Abl Subcellular Localization (Table I)

1. Activated Abl Is Cytoplasmic

The mouse Abl gene was discovered as the normal cellular homolog of v-Abl, the oncogene of Abelson murine leukemia virus (A-MuLV). Human ABL is fused with BCR (break point cluster region) through chromosomal translation and the BCR-ABL oncogene is responsible for the development and progression of chronic myelogenous leukemia (CML). The v-Abl and BCR-ABL oncoproteins are autophosphorylated on tyrosines and exhibit constitutively activated kinase activity. The three NLS and the NES are intact in v-Abl and BCR-ABL. However, both oncoproteins are localized exclusively to the cytoplasm. BCR-ABL does not accumulate in the nucleus even when nuclear
Abl NES activity is stronger than the Abl NLS. (A) Schematics of GFP–Abl fusion constructs. A segment of Abl (murine Abl aa 610 to 793) encompassing the three NLS was fused to GFP to create the fusion construct GFP-(Abl)NLS. The Abl NES (murine Abl aa 1094 to 1114) was added to the C terminus of the GFP-(Abl)NLS to make GFP-(Abl)NLS + NES. (B) Subcellular distribution of GFP-(Abl)NLS and GFP-(Abl)NLS + NES. The two GFP-tagged constructs were transiently expressed in 293T cells. The GFP images were captured with a fluorescence microscope. GFP-(Abl)NLS is exclusively nuclear, showing the Abl NLS to be functional in driving the import of a reporter protein. GFP-(Abl)NLS + NES is exclusively cytoplasmic, showing the NES to be stronger than the three Abl NLS.
export is deactivated with LMB (Vigneri and Wang, 2001), suggesting its NLS is defunct. However, inhibition of BCR-ABL kinase with STI571 can activate its nuclear import (Vigneri and Wang, 2001). Thus, an inverse correlation exists between Abl kinase activity and its nuclear import.

BCR-ABL acquires the coiled-coil tetramerization domain of BCR, and oligomerization is responsible for the persistent autophosphorylation. A crystal structure of the Abl kinase domain indicates that phosphorylation at Tyr-245 in the SH2–kinase linker and at Tyr-412 in the activation loop can circumvent the autoinhibitory mechanism of Abl (Hantschel et al., 2003; Harrison, 2003; Nagar et al., 2003). Thus, autophosphorylated BCR-ABL is not likely to adopt the autoinhibited conformation. This raises two possible reasons for the inhibition of Abl NLS in BCR-ABL. Is it the kinase activity per se or is it the activated conformation that causes the loss of nuclear import?

We favor the notion that nuclear import is blocked by the active conformation of BCR-ABL, based on the following observations. The kinase activity of BCR-ABL can be inhibited by mutation of the critical lysine at the ATP-binding site. The resultant mutant, BCR-ABL(KD) (kinase defective), forms oligomers without autophosphorylation. Unlike BCR-ABL, BCR-ABL(KD) could be accumulated in the nucleus of cells treated with LMB (Vigneri and Wang, 2001), suggesting the NLS recovers its function in BCR-ABL(KD). However, no more than 30% of the total pool of BCR-ABL(KD) ended up in the nucleus irrespective of the duration of LMB treatment (Vigneri and Wang, 2001). The majority (~70%) of BCR-ABL(KD) remained defective for nuclear import. Therefore, autophosphorylation of BCR-ABL is not the only cause of nuclear import inhibition. Instead, autophosphorylation may lock BCR-ABL in an active conformation, in which the three NLS are inaccessible for nuclear import. BCR-ABL(KD) lacks the locking mechanism, hence a fraction of it (~30%) might transit out of the active conformation and become imported. The majority of BCR-ABL(KD), however, would remain in a conformation that is incompatible with nuclear import. In keeping with this idea, ST1571 has been shown to force BCR-ABL into the autoinhibited conformation (Azam et al., 2003; Smith et al., 2003). Taken together, the current evidence suggests Abl NLS to be functional only when Abl kinase adopts the autoinhibited conformation.

Autophosphorylation of Abl is observed in vitro or on excessive overproduction in transiently transfected cells. However, Abl rarely attains the autophosphorylated conformation under physiological conditions, possibly because Abl does not form stable oligomers and/or it is highly sensitive to the action of tyrosine phosphatases. Locking Abl out of the active conformation may be required for its nuclear import. The proposed conformational regulation of Abl NLS would suggest a gating mechanism that avoids import of already activated Abl kinase into the nucleus.
2. Cell Adhesion Stimulates Abl Export

In fibroblasts, adhesion to extracellular matrix (ECM) can stimulate a transient wave of Abl nuclear export that is dependent on the Abl NES (Table I). This rapid exodus is observed during the inceptive phase of integrin-mediated cell adhesion to fibronectin, vitronectin, or collagen (Lewis et al., 1996). Corresponding to this wave of export, Abl also joins the early focal adhesion complexes. The Abl pool reequilibrates between the cytoplasmic and nuclear compartments after cells spread onto the substratum, coincident with the subsidence of Abl at the more mature focal adhesions (Lewis et al., 1996). Integrin-mediated cell adhesion also causes a transient activation of Abl kinase activity. The adhesion-dependent pulsation of Abl shuttling is observed with the Abl(KD) mutant, suggesting that Abl kinase activity per se is not required for its export and reimport (Lewis et al., 1996). How adhesion signals reach the nuclear pool of Abl to stimulate its rapid export has remained a mystery.

3. Caspase Cleavage Prevents Abl Nucleocytoplasmic Shuttling

Caspases are cysteine proteases activated by inflammatory cytokines, such as FAS ligand and tumor necrosis factor (TNF), or by the mitochondria-dependent apoptosome (Shi, 2002; Thornberry, 1997). Activation of caspases occurs mostly in the cytoplasm. Three caspase cleavage sites have been discovered in the C-terminal region of Abl (Figs. 1 and 3). Cleavage at the most C-terminal of these sites disrupts the actin-binding function and removes the NES of Abl. As expected, a recombinant Abl mimicking truncation at this C-terminal cleavage site is accumulated in the nucleus (Barila et al., 2003). Cleavage at the next site from the C terminus will remove NLS-2, NLS-3, and the NES but spare NLS-1 (Fig. 1). This cleaved Abl fragment would also be accumulated in the nucleus under conditions in which the NLS-1 is functional. Cleavage at the most N-terminal of the three sites would remove all NLS and NES, leaving a fragment of Abl that is trapped in the cytoplasm. Thus, caspase cleavage of Abl in the cytoplasm is expected to cause a shift in the subcellular distribution of Abl and to block the nucleocytoplasmic shuttling of Abl kinase (Table I).

4. Myogenic Differentiation Inhibits Abl Nuclear Import

Abl tyrosine kinase is expressed in proliferating and differentiated cells. Although differentiation does not affect Abl expression, it regulates Abl subcellular distribution. In cultured myoblasts, Abl is distributed between the cytoplasm and the nucleus. Treatment of myoblasts with LMB causes nuclear accumulation of Abl, demonstrating that Abl undergoes nuclear
import and export in myoblasts. In differentiated myotubes, however, Abl becomes exclusively cytoplasmic (Puri et al., 2002). Treatment of myotubes with LMB does not cause nuclear accumulation of Abl, suggesting a block in Abl nuclear import (Table I). Myoblast differentiation in culture is induced by serum withdrawal and cell–cell contact. We have found that cell–cell contact or serum withdrawal can shift Abl into the cytoplasm of fibroblast cells. However, LMB treatment of quiescent fibroblasts still causes the nuclear accumulation of Abl protein (Taagepera and Wang, unpublished observation). Therefore, the inhibition of Abl nuclear translocation is not associated with quiescence but most likely is the result of terminal differentiation.

### III. Cytoplasmic Function of Abl

#### A. Abl Interaction with Cell Surface Receptors

Cytoplasmic tyrosine kinases are important transducers of extracellular signals. Abl is no exception. In the cytoplasm, Abl transduces extracellular signals to regulate F-actin (Woodring et al., 2003). A variety of cell surface receptors have been reported to interact with Abl. These include receptors for the ECM, growth factors, and neurotransmitters (Table II). Abl kinase activity is activated by cell adhesion to the ECM or by platelet-derived growth factor (PDGF) (Lewis et al., 1996; Plattner et al., 1999, 2003). However, Abl does not physically associate with the ECM receptors (integrins) or the PDGF receptor. Interestingly, Abl does associate with several receptors in neurons, including the NGF receptor, the Ephrin receptors, and the NMDA receptor (Glover et al., 2000; Yano et al., 2000; Yu et al., 2001). At present, there is no direct evidence showing activation of Abl kinase by NGF, Ephrin, or NMDA in neurons. Instead, the NMDA receptor associates with Abl to inhibit its kinase activity. In ECM- or
PDGF-stimulated cells, Abl kinase plays a role in regulating F-actin dynamics and mitogenic signal transduction.

B. Negative Regulation of Cell Spreading and Cell Migration by Abl

Cell spreading and cell migration on ECM are regulated by integrins, the receptors for ECM proteins (Table II). In fibroblasts, integrin-mediated cell adhesion to fibronectin triggers a surge of Abl kinase activity, which tapers off as cells spread on the substratum (Lewis et al., 1996). Activation of Abl kinase correlates with the transient colocalization of Abl with integrins to the early focal contacts (Lewis et al., 1996). A consequence of Abl activation is the slowing of cell spreading on fibronectin. Fibroblasts derived from Abl-null mouse embryos spread faster than their wild-type counterparts (Frasca and Wang, unpublished data). Restoration of Abl expression in the Abl-null fibroblasts reduced the rate of spreading, and this reduction required Abl kinase activity. The faster spreading of Abl-null fibroblasts is correlated with the extension of lamellipodia along the entire circumference, a morphology resembling a “fried egg” or “pancake.” This morphology is even more pronounced with fibroblasts from Abl/Arg double-knockout mice (Woodring et al., 2002). Restoration of Abl kinase disrupts this morphology, allowing tassels of F-actin “microspikes” to protrude from the spreading cells (Koleske et al., 1998). The effect of Abl on cell spreading is transient, occurring between 10 and 20 min after plating cells onto fibronectin. At steady state, Abl-null or Abl/Arg double-knockout fibroblasts do not exhibit significantly altered morphology from their wild-type counterpart.

Abl kinase also exerts a negative effect on cell migration, a process that is critically dependent on cell–matrix interaction (Table II). Inhibition of Abl kinase is correlated with increased chemotaxis response to hepatocyte growth factor (HGF) in thyroid carcinoma cells (Frasca et al., 2001). Transient coexpression of Abl reduces the motile response stimulated by the overproduction of p130Cas, most likely by disrupting the formation of a p130Cas–Crk complex (Kain and Klemke, 2001). At present, it is not clear whether Abl also inhibits cell spreading by a negative regulation of the p130Cas–Crk complex.

Negative regulation of cell spreading or cell migration is likely to play important roles in development, tissue remodeling, and wound repair. The biological purpose for Abl-dependent slowing of cell spreading or cell migration may not be elucidated by in vitro experiments with isolated fibroblasts. To fully understand the biological role of Abl in ECM-regulated cellular processes may require tools that can measure cell spreading or cell migration in vivo in real time. Applying such tools to the Abl-null mice may help to explain some of the pleiotropic phenotypes.
Stimulation of fibroblasts with platelet-derived growth factor (PDGF) also causes a transient activation of Abl kinase activity (Plattner et al., 1999, 2003) (Table II). PDGF receptor kinase autophosphorylates on multiple tyrosine sites, each of which recruits a different cytoplasmic effector to transduce the PDGF signal. Direct interaction of Abl with activated PDGF receptor has not been reported. Instead, Abl kinase activation requires the recruiting of two different cytoplasmic effectors by the PDGF receptor (Fig. 3). One of the effectors is the Src family of nonreceptor tyrosine kinases (Plattner et al., 1999). The other is phospholipase C-γ1 (PLC-γ1) (Plattner et al., 2003). The Src kinase may activate Abl by phosphorylating it at Tyr-412 to disrupt the autoinhibited conformation. How phospholipase C contributes to Abl activation is not fully understood, but might involve the removal of phospholipids that can inhibit Abl kinase (Plattner et al., 2003).

PDGF causes a major remodeling of the F-actin structure, because it is both a growth factor and a chemoattractant for fibroblasts. On exposure to PDGF, fibroblasts elaborate circular ruffles on their surface and this response is compromised, albeit not abolished, in Abl-null fibroblasts (Plattner et al., 1999). The PDGF-stimulated ruffles can serve two purposes, either to form a leading edge for motility or to stimulate nutrient uptake for cell growth. The reduction of ruffles in Abl-null fibroblasts may therefore compromise the growth or the motile response to PDGF. As discussed above, Abl kinase inhibits the motile response to HGF in thyroid cancer cells (Frasca et al., 2001). We have also observed that cytoplasmic Abl kinase inhibits the motile response of mouse embryo fibroblasts to insulin-like growth factor type I (IGF-I) (Fransca and Wang, unpublished results). On the other hand, Abl kinase has been shown to play a positive role in PDGF-dependent activation of Myc (Furstoss et al., 2002). It thus appears that Abl may contribute to mitogenic signal transduction downstream of PDGF receptor activation, possibly by diverting the signaling machinery away from the motile response to PDGF.

IV. Nuclear Function of Abl

A. Negative Regulators of Nuclear Abl Kinase

As discussed above, nuclear import may be restricted to Abl protein of an inactive conformation. This is because constitutively active oncogenic v-Abl and BCR-ABL are exclusively cytoplasmic. Once imported, inhibition
Figure 3  Cytoplasmic and nuclear functions of Abl. Abl kinase is activated by cell adhesion mediated by integrin, or by PDGF mediated by the PDGF receptor. The mechanism of Abl activation by cell adhesion is not understood. The inactivation of Abl kinase in detached cells requires Abl interaction with F-actin (Woodring et al., 2001). F-actin can inhibit Abl kinase in vitro, raising the possibility that cell adhesion may override the inhibitory effect of F-actin to activate Abl kinase (Woodring et al., 2001, 2003). Activation of Abl kinase by PDGF requires the recruitment of Src and PLCγ1 to the activated PDGF receptor. The Src tyrosine kinase may directly phosphorylate Abl (Plattner et al., 1999). PLCγ1 may degrade phospholipids that mediate the inhibition of Abl kinase (Plattner et al., 2003). The current evidence suggests activated Abl tyrosine kinase to be excluded from the nucleus; only the autoinhibited Abl conformation would allow the Abl NLS to be accessible for nuclear import. In the nucleus, Abl is kept latent through its association with RB or BRCA1. RB dissociates from Abl on its phosphorylation by Cdk/cyclin or its degradation by caspase. BRCA1 dissociates from Abl on
of nuclear Abl kinase is further enforced by interaction with other nuclear proteins. The retinoblastoma tumor suppressor protein (RB) (Welch and Wang, 1993, 1995), and the breast cancer 1 tumor suppressor (BRCA1) (Foray et al., 2002), are two known inhibitors of nuclear Abl kinase (Table II).

1. RB Blocks Signaling to Nuclear Abl Kinase

RB binds to the Abl kinase domain and prevents Abl from being activated by other signals in the nucleus. RB can be inactivated by phosphorylation or degradation (Chau and Wang, 2003). Phosphorylation and/or degradation of RB are required to release nuclear Abl and permit its activation.

One of the signals that consistently activates nuclear Abl kinase is DNA damage (Wang, 2000). Exposing cells to genotoxic agents causes activation of the nuclear pool of Abl tyrosine kinase. DNA damage-dependent activation of nuclear Abl kinase requires the release of Abl from RB. DNA damage does not activate nuclear Abl in G0/G1 cells that contain a functional RB (Liu et al., 1996). However, DNA damage can activate nuclear Abl in S-phase cells (Liu et al., 1996).

Another signal that activates the nuclear Abl kinase is tumor necrosis factor α (TNF) (Dan et al., 1999; Le et al., 1998). As discussed above, TNF activates caspase to cleave Abl in the C-terminal region (Barila et al., 2003). Two of the cleaved Abl fragments retain at least one of the NLS but lack the NES, hence they can accumulate in the nucleus. Cleavage by caspase is not sufficient to activate Abl kinase (Barila et al., 2003). We have found that activation of nuclear Abl kinase by TNF also requires the degradation of RB. We have created a caspase-resistant Rb-MI allele in mice (Chau et al., 2002). Rb-MI cells are resistant to TNF receptor (TNFR)-induced apoptosis. Interestingly, nuclear Abl kinase is activated by TNF in Rb+/+ and Rb−/− cells, but this activation is blocked in RbMI/MI cells (Chau and Wang, unpublished data). These results suggest that RB degradation is necessary for activation of Abl kinase by TNF

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for TNF to activate Abl. However, RB degradation is not the only factor in Abl activation. Another unknown factor also contributes to the increase in nuclear Abl kinase activity. Together, these results suggest that TNF regulates Abl at several levels, mediated in part by caspase cleavage of Abl itself and of RB.

### 2. BRCA1 Releases Abl in DNA Damage Signaling

BRCA1-associated Abl is also inactive. Disruption of BRCA1–Abl interaction is observed on DNA damage, correlating with phosphorylation of BRCA1 and Abl by the ATM kinase (Foray et al., 2002). Disruption of BRCA1–Abl interaction also correlates with the activation of Abl tyrosine kinase. Interestingly, BRCA1 expression is upregulated at each G1/S
transition (Scully et al., 1997). It is conceivable that BRCA1 may replace RB to control the activation of Abl by DNA damage in S/G2 cells.

B. ATM and Related Protein Kinases Activate Nuclear Abl Kinase

Central to DNA damage signaling is a family of conserved protein kinases belonging to the phosphatidylinositol 3-kinase (PI3K) superfamily of kinases. In mammalian cells, at least four PI3K-like protein kinases are involved in DNA damage response, and they are ATM (ataxia telangiectasia mutated), ATR (ATM-related), ATX (ATM and ATR related), and DNA-PK (DNA-dependent protein kinase) (Abraham, 2001). The PI3K-like protein kinases phosphorylate their substrates at a simple motif of S-Q or T-Q. In c-Abl, an S-Q motif (Ser-465 to Gln-466) is present in the kinase domain; this S-Q motif can be phosphorylated in vitro by ATM or DNA-PK (Baskaran et al., 1997; Kharbanda et al., 1997) (Table II). Mutation of Ser-465 to alanine abolished in vitro phosphorylation of Abl by ATM. The S465A mutant cannot be activated by ionizing radiation or cisplatin in cells. Mutation of Ser-465 to glutamate, mimicking phosphorylation, increases the basal activity of Abl kinase (Baskaran et al., 1997). Taken together, these results suggest that nuclear Abl is a substrate of the PI3K-like protein kinases, and the phosphorylation of Abl at Ser-465 is required for its activation by DNA damage.

C. Nuclear Abl Regulates DNA Damage Response

1. Interaction between Abl and DNA Repair Proteins

Nuclear Abl interacts with several proteins involved in DNA repair (Table II). The afore mentioned BRCA1, ATM, and DNA-PK can each regulate or conduct DNA repair. BRCA1 and ATM are involved in homology recombination repair (HRR) of double-stranded breaks (D’Andrea and Grompe, 2003; Wu et al., 2000). DNA-PK is important for nonhomologous end joining (NHEJ) repair of double-stranded breaks (Durocher and Jackson, 2001; Gellert, 2002). Abl has been reported to interact with RAD51 and RAD52 (Chen et al., 1999; Kitao and Yuan, 2002; Yuan et al., 1998, 2003), which are also involved in HRR. Abl also interacts with UV-DDB, a protein involved in the repair of ultraviolet (UV) damage (Cong et al., 2002), although UV irradiation does not activate the nuclear Abl kinase. The mismatch repair system, on the other hand, is required for cisplatin to activate the Abl kinase (Gong et al., 1999; Nehme et al., 1997). Despite its interaction with repair proteins, Abl does not appear to have an essential role in DNA repair per se. This is because Abl-null cells do
not exhibit the classic phenotype of repair-deficient cells, that is Abl-null cells are not hypersensitive to ionizing radiation, UV, or cisplatin (Baskaran et al., 1997). Abl-null cells also do not exhibit defects in mismatch repair, that is, microsatellite instability (Baskaran et al., 1997; Takao et al., 2000). If Abl does not participate in the repair process, what then is the purpose of its interaction with repair proteins? A likely explanation may be that these interactions allow Abl to “sense” the progress of DNA repair so that it can “transduce” a repair-dependent signal to regulate cellular responses that are downstream of DNA repair.

2. Functional Interaction between Abl and p53

The p53 tumor suppressor protein is an important transducer of DNA damage signal. Activation of p53 stimulates the transcription of many genes to regulate cell cycle progression, DNA repair, and apoptosis (Levine, 1997; Vousden, 2000). Functional interaction between Abl and p53 has been reported to occur in two ways.

The C-terminal region of human Abl appears to bind p53 and this interaction correlates with a modest enhancement of transcription from p53-dependent promoter in transient cotransfection experiments (Nie et al., 2000; Yuan et al., 1996). Enhancement of transcription by Abl has also been observed from other promoters, including the human immunodeficiency virus (HIV) long terminal repeat (LTR), which is dependent on the HIV Tat protein for transcription. With the HIV LTR, Abl can replace Tat to stimulate the elongation phase of transcription. This effect of Abl is mediated through tyrosine phosphorylation of the C-terminal repeated domain of RNA polymerase II (Baskaran et al., 1999). Therefore, the transcription enhancement function of Abl may not be restricted to p53-regulated promoters.

The Abl kinase has been shown to phosphorylate Mdm2, which negatively regulates p53 function. Mdm2 associates with p53 to inhibit the transactivation function of p53 and to facilitate the nuclear export and polyubiquitination of p53 for proteosome-mediated degradation (Chene, 2003). Phosphorylation of Mdm2 by Abl is correlated with the ability of Abl to rescue p53 from proteosomes (Goldberg et al., 2002; Sionov et al., 1999). Thus, Abl can exert a positive effect on p53 by antagonizing Mdm2.

Cells lacking p53 are defective in DNA damage response. Depending on the cell type and the damage inducers, p53 either causes cell cycle arrest or apoptosis. Unlike p53-deficient cells, Abl-null cells can be arrested in G₁ or G₂ after DNA damage (Baskaran et al., 1997). Abl-null cells do show reduced apoptosis response to DNA damage; however, Abl-mediated activation of apoptosis can occur in p53-null cells (Vella et al., 2003). On the other hand, overproduction of nuclear Abl protein can inhibit cell proliferation and this is dependent on p53 (Sawyers et al., 1994; Wen et al.,
Taken together, these results suggest Abl can activate p53 function when it is overproduced in the nucleus. However, Abl is not required for p53 activation under experimental conditions in which cells are overwhelmed with DNA damage. Nevertheless, Abl may modulate the efficiency or the duration of p53 function under certain physiological conditions that have yet to be recapitulated in cultured cells.

3. Functional Interaction between Abl and p73

The p53-related p73 protein also regulates cellular response to DNA damage. Functional interaction between Abl and p73 plays an important role in DNA damage-induced apoptosis. The p73 protein is stabilized and activated through an Abl-dependent mechanism in damaged cells (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). Stabilization of p73 is not mediated by the inhibition of Mdm2, because Mdm2 does not cause the degradation of p73 (Balint et al., 1999; Zeng et al., 1999). Regulation of p73 protein stability may involve its interaction with PMS2, a component of the mismatch repair system (Gong et al., 1999). The precise mechanism for DNA damage-induced stabilization of p73 is currently not understood.

The Abl SH3 domain can bind to a PXXP motif in p73 and phosphorylates p73 at Tyr-99 and possibly other sites. Substitution of Tyr-99 in p73 with phenylalanine reduces the apoptotic function of p73 (Agami et al., 1999). In addition, Abl is required for p73 to be acetylated at three lysine residues by the transcription coactivator, p300 (Costanzo et al., 2002). Substitution of these three lysines with arginines also compromises the apoptotic function of p73 (Costanzo et al., 2002). The Abl-dependent acetylation of p73 by p300 suggests a possible three-way interaction between these proteins. However, the precise mechanisms of p73 regulation by Abl and p300 remain to be elucidated.

Fibroblasts from p73-knockout mouse embryos showed reduced apoptosis response to DNA damage inducers, such as Adriamycin or ionizing radiation (Flores et al., 2002). Restoration of p73α expression in p73-null cells increased the sensitivity to Adriamycin (Ki and Wang, unpublished data). These results show that p53 and p73 each contribute to the apoptotic response to DNA damage.

To enforce the nuclear accumulation of a regulatable Abl, we have constructed Abl–Nuk–FKBP, which is a constitutively nuclear Abl activatable through FKBP-dependent dimerization (Vella et al., 2003). This Abl–Nuk–FKBP protein contains (1) the L1067A mutation in the NES to prevent export, (2) three tandem copies of the simian virus 40 (SV40) NLS to enforce import, and (3) two copies of the FK506-binding protein for ligand-induced dimerization. Ectopic expression of Abl–Nuk–FKBP caused dimerizer-dependent apoptosis (Vella et al., 2003). This nuclear Abl-induced
apoptosis occurred in p53-null cells, but not in p73-null cells. Cotransfection of Abl–Nuk–FBKP with p73 rescued the apoptotic response in p73-null cells (Vella et al., 2003). Thus, Abl and p73 function in the same pathway toward apoptosis. Studies performed in our laboratory have found that Abl and p73 also participate in TNF-induced apoptosis, which is completely independent of p53 (Chau et al., in press, 2004). Moreover, Abl and p73 account for the augmentative effect of cell adhesion on the sensitivity to DNA damage (Truong et al., 2003). Therefore, current evidence suggests that p53 and p73 function in two parallel proapoptotic pathways. The nuclear Abl kinase can activate both p53 and p73; moreover, Abl is an essential activator of p73-dependent apoptosis.

4. Abl Inhibits Myogenic Differentiation

DNA damage triggers checkpoints to prevent cell cycle progression and allow time for DNA repair. We have found that DNA damage also prevents the commitment to differentiate, delaying global reprogramming of gene expression until DNA is repaired (Puri et al., 2002). This “differentiation checkpoint” was observed in cultured myoblasts on exposure to sublethal doses of damage inducers. Cultured myoblasts express the myogenic transcription factor, MyoD, in a latent form. Activation of MyoD can be triggered by the withdrawal of mitogens. In combination with high cell density, MyoD activates muscle-specific gene expression and induces cell fusion to form multinucleated myotubes. If myoblasts are challenged with DNA damage inducers and then placed under differentiation conditions, muscle-specific gene expression is inhibited as long as the damage inducers are present (Puri et al., 2002). Muscle gene expression can be restored on removal of damaging agents. The inhibition of muscle gene expression is not observed if already differentiated myotubes are exposed to the same genotoxic drugs (Puri et al., 2002). Thus, DNA damage causes a reversible inhibition of the commitment to differentiate, but does not affect muscle gene expression once differentiation has occurred.

Inhibition of muscle gene expression by DNA damage can occur in the absence of p53. However, this differentiation checkpoint is compromised in Abl-null cells (Puri et al., 2002). The MyoD transcription factor appears to be a target of Abl kinase. Abl associates with MyoD in transient coexpression experiments, and phosphorylates MyoD at two tyrosine sites in vitro. Mutation of the N-terminal tyrosine, which is conserved in the MyoD protein of several species, does not interfere with the activation of muscle gene expression. The YF-mutated MyoD can transactivate muscle-specific promoters. The transactivation function of MyoD is reduced by DNA damage. However, the transactivating function of YF-mutated MyoD is not inhibited by DNA damage (Puri et al., 2002).
These results suggest that tyrosine phosphorylation of MyoD by Abl contributes to DNA damage-induced inhibition of muscle gene expression.

In summary, the nuclear Abl tyrosine kinase activity is regulated by negative and positive factors. Activated Abl can regulate transcription through its interaction with transcription factors and RNA polymerase II. Abl also interacts with repair proteins, suggesting it may transduce DNA repair-dependent signals to regulate gene expression. Activation of nuclear Abl kinase by DNA damage can either cause an inhibition of differentiation or an activation of apoptosis. The status of DNA repair may dictate which of the two functions of Abl are manifested in a damaged cell. Accumulation of irreparable lesions in the genome may shift Abl from its role as a reversible regulator of differentiation to that of an irreversible inducer of apoptosis.

V. Nuclear Exclusion of BCR-ABL or Abl in Cancer Cells

A. Chronic Myeloid Leukemia

1. BCR-ABL Tyrosine Kinase Causes CML

As discussed above, oncogenic BCR-ABL of CML resides in the cytoplasm of leukemic cells. The autophosphorylated BCR-ABL exploits a gating mechanism that prevents nuclear import of kinase-activated Abl. By excluding itself from the nucleus, BCR-ABL may avoid the proapoptotic function of activated nuclear Abl kinase.

In the cytoplasmic sanctuary, autophosphorylated BCR-ABL binds to SH2 domains of various adaptor proteins to activate a number of signaling pathways, including the Ras/MAP kinase cascade, JNK, STATs, PI3K/Akt, and NF-κB (Danial and Rothman, 2000; Turhan et al., 1998). BCR-ABL also weakens cell adhesion to the ECM, stimulates cell motility, and causes an increase in cellular reactive oxygen species (ROS) (Feller, 2001; Salesse and Verfaillie, 2002; Salgia et al., 1997, 1999; Sattler et al., 2000; Turner, 2000; Wertheim et al., 2002). The end result of the promiscuous activity of BCR-ABL is increased proliferation, decreased apoptosis, and a predisposition to genetic instability. The BCR-ABL kinase activity is of critical importance in CML, because an inhibitor of this kinase, that is, GLEEVEC, has successfully controlled this leukemia (Druker, 2001; Druker et al., 2001a,b).

2. CML Therapy with GLEEVEC

Treatment with GLEEVEC has caused remission in ~90% of patients in chronic-phase CML. However, BCR-ABL-positive cells are not completely eradicated by GLEEVEC even in patients with clinically satisfactory
remission. In CML blast crisis, the hematological response to GLEEVEC is usually short-lived because of the development of drug resistance. Amplification of the BCR-ABL gene and mutations in BCR-ABL protein are the two most common mechanisms of resistance to GLEEVEC (Druker, 2001; Druker et al., 2001a,b). Therefore, the challenge facing CML therapy is to enhance the efficacy of GLEEVEC so that BCR-ABL-positive cells in chronic phase and blast crisis can be quickly eliminated. A rapid eradication of BCR-ABL-positive cells would mitigate the emergence of GLEEVEC-resistant clones.

3. Nuclear Entrapment of BCR-ABL Kills CML Cells

GLEEVEC (STI571) interacts with the ATP-binding site in the Abl kinase domain. The crystal structure of STI571 in complex with Abl kinase domain has revealed that this drug has a profound effect on the Abl kinase conformation, causing the activation loop to fold back onto the kinase C lobe, thereby locking Abl in an inactive conformation (Schindler et al., 2000). The STI571-induced inactivated conformation requires intramolecular interactions between the Abl SH3, SH2, and kinase domains (Azam et al., 2003; Nagar et al., 2003; Smith et al., 2003). As a result of this conformational change, STI571 not only inhibits BCR-ABL kinase but also revives its NLS function (Vigneri and Wang, 2001). As discussed above, the NES of Abl can override the activity of the three NLS (Fig. 2). Therefore, STI571 alone does not cause nuclear accumulation of BCR-ABL. However, when leukemia cells are treated with a combination of STI571 and LMB, BCR-ABL accumulates in the nucleus (Vigneri and Wang, 2001).

The inhibitory effect of STI571 on BCR-ABL kinase is reversible. This reversibility and the rapid clearing of this drug in vivo necessitate the continuous administration of GLEEVEC. The inhibitory effect of LMB on exportin 1 is irreversible. Recovery from LMB necessitates the de novo synthesis of exportin 1. Therefore, on removal of both drugs, the differing pharmacokinetics allow a window of time during which BCR-ABL is retained in the nucleus but regains its kinase activity. Interestingly, reactivation of BCR-ABL kinase in the nucleus causes the activation of apoptosis, leading to a complete and irreversible eradication of leukemia cells (Vigneri and Wang, 2001). This result provides the most vivid illustration for location-dependent function of Abl kinase. In the cytoplasm, the hyperactivity of BCR-ABL leads to vigorous proliferation. In the nucleus, the hyperactivity of BCR-ABL issues the command for cellular suicide. Ironically, the BCR-ABL oncoprotein is also the Achilles’ heel of CML cells. The latent NLS function of BCR-ABL affords an opportunity to convert this oncoprotein into a dagger at the heart of CML cells. Whether the combination
of STI571 and LMB can be used to eradicate CML cells in patients awaits further investigation.

B. Anaplastic Thyroid Cancer

1. Nuclear Import of Abl Is Compromised in Anaplastic Thyroid Cancer

Another example of Abl being excluded from the nucleus has been found in cells derived from anaplastic thyroid cancer (ATC), the most aggressive and deadly form of thyroid carcinoma (Vella et al., 2003). In normal thyroid epithelial cells, and in cells derived from papillary or follicular thyroid cancer, ABL is mostly cytoplasmic but can accumulate in the nucleus upon LMB treatment. Thus, ABL undergoes nucleocytoplasmic shuttling in these cells. The ABL protein is also cytoplasmic in cells derived from ATC, but ABL fails to accumulate in the nucleus on LMB treatment (Vella et al., 2003). Therefore, nuclear import of ABL is disrupted in ATC cells. This would imply that ABL might adopt an active conformation in the cytoplasm of ATC cells. The exact mechanism for ABL cytoplasmic retention in ATC cells is presently unknown.

2. Subcellular Segregation of Abl from p73

Another property of ATC cells, not shared by normal thyroid cells or papillary and follicular thyroid cancer cells, is the expression of p73α. The upregulation of full-length p73α is observed in ATC cell lines and in primary ATC samples (Vella et al., 2003). The upregulation of p73 in cancer cells has been described not only for ATC, but also in breast, bladder, and liver cancers (Levrero et al., 2000; Melino et al., 2002). The increased expression of full-length p73α in cancer cannot be explained by the current evidence that p73α is functionally similar to the p53 tumor suppressor protein. Perhaps p73α has other functions that can contribute to tumor development. In this respect, p63, a closer relative of p73, has been proposed to maintain the epithelial stem cells that replenish the skin. If p73α can also maintain the stem cell fate, it could conceivably contribute to tumor formation. In ATC cells, p73α is localized to the nucleus, separated from ABL. As would be expected from the interdependence of ABL and p73 in activating apoptosis, enforced activation of a nuclear Abl (Abl–Nuk–FKBP) caused p73-dependent death of ATC cells (Vella et al., 2003). The nuclear exclusion of ABL in ATC cells indicates that subcellular segregation of p73 from ABL may restrain the p73 tumor suppressor function to facilitate tumor development. Nuclear exclusion of ABL in ATC cells suggests that exploitation of the
cytoplasmic Abl function may not be restricted to CML. It will be of interest to determine whether the nucleocytoplasmic shuttling of ABL is compromised in other human cancer cells.

VI. Conclusions and Future Prospects

Current evidence supports the conclusion that Abl subcellular localization dictates its biological effects. In the cytoplasm, Abl regulates F-actin and promotes mitogenic signaling in response to ECM and growth factors. In the nucleus, Abl inhibits differentiation and induces apoptosis in response to DNA damage or death receptor signals. The BCR-ABL oncoprotein resides exclusively in the cytoplasm and causes chronic myelogenous leukemia. When trapped in the nucleus, BCR-ABL kinase behaves like an antioncoprotein by activating the suicide program to kill leukemic cells. The ABL protein is excluded from the nucleus of anaplastic thyroid cancer cells. Enforced nuclear accumulation and activation of Abl kinase also induces p73-dependent apoptosis in these cancer cells.

An interesting theme has begun to emerge concerning the regulation of Abl localization. Signals that promote cell survival appear to stimulate Abl nuclear export or retain Abl in the cytoplasm. One example is the ECM-stimulated export of Abl during the initial phase of cell adhesion. Another example is the cytoplasmic retention of Abl in terminally differentiated myotubes, which are highly resistant to DNA damage-induced apoptosis. On the other hand, signals that promote apoptosis appear to stimulate Abl nuclear accumulation. For example, activated caspase can cleave off the Abl NES to retain Abl in the nucleus.

Taken together, the current results are consistent with a simple model: nuclear accumulation of activated Abl tyrosine kinase causes cell death; nuclear export or inhibition of nuclear import prevents Abl from killing cells. Therefore, death by Abl is regulated by the subcellular location of this tyrosine kinase. This model does not exclude the possibility that cytoplasmic Abl may contribute to apoptosis; this is because the activated nuclear Abl can conceivably be exported to the cytoplasm to stimulate events associated with apoptotic cell death.

At present, we do not fully understand how nucleocytoplasmic shuttling of Abl is regulated. The observation that activated Abl kinase cannot enter the nucleus is interesting and does not follow the paradigm for activation-dependent nuclear import of MAP kinases such as ERK (Ben-Levy et al., 1998; Reiser et al., 1999). However, if nuclear accumulation of activated Abl kinase is to trigger cell death, it is reasonable for cells to import only the inactive form of Abl kinase at steady state. The rapid nuclear export of Abl may provide another safeguard against premature commitment to
suicide. Understanding the regulation of Abl nuclear import and export will shed light on how to control the biological outputs from the capable Abl tyrosine kinase.

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7. Death by Abl: A Matter of Location


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