Chapter 1
Overview: What Are Helicases?

Colin G. Wu and Maria Spies

Abstract First discovered in the 1970s, DNA helicases were initially described as enzymes that use chemical energy to separate (i.e., to unwind) the complementary strands of DNA. Because helicases are ubiquitous, display a range of fascinating biochemical activities, and are involved in all aspects of DNA metabolism, defects in human helicases are linked to a variety of genetic disorders, and helicase research continues to be important in understanding the molecular basis of DNA replication, recombination, and repair. The purpose of this book is to organize this information and to update the traditional view of these enzymes, because it is now evident that not all helicases possess bona fide strand separation activity and may function instead as energy-dependent switches or translocases. In this chapter, we will first discuss the biochemical and structural features of DNA—the lattice on which helicases operate—and its cellular organization. We will then provide a historical overview of helicases, starting from their discovery and classification, leading to their structures, mechanisms, and biomedical significance. Finally, we will highlight several key advances and developments in helicase research, and summarize some remaining questions and active areas of investigation. The subsequent chapters will discuss these topics and others in greater detail and are written by experts of these respective fields.

DNA: The Source of Genetic Information

DNA is the "blueprint of life" and stores within the necessary instructions for living cells to grow and to function. The existence of DNA has been known since 1869. It took, however, almost a century to discern DNA structure and its role in the

C.G. Wu • M. Spies (✉)
Department of Biochemistry, University of Iowa Carver College of Medicine,
Iowa City, IA, USA
e-mail: maria-spies@uiowa.edu

M. Spies (ed.), *DNA Helicases and DNA Motor Proteins*, Advances in Experimental Medicine and Biology, DOI 10.1007/978-1-4614-5037-5_1,

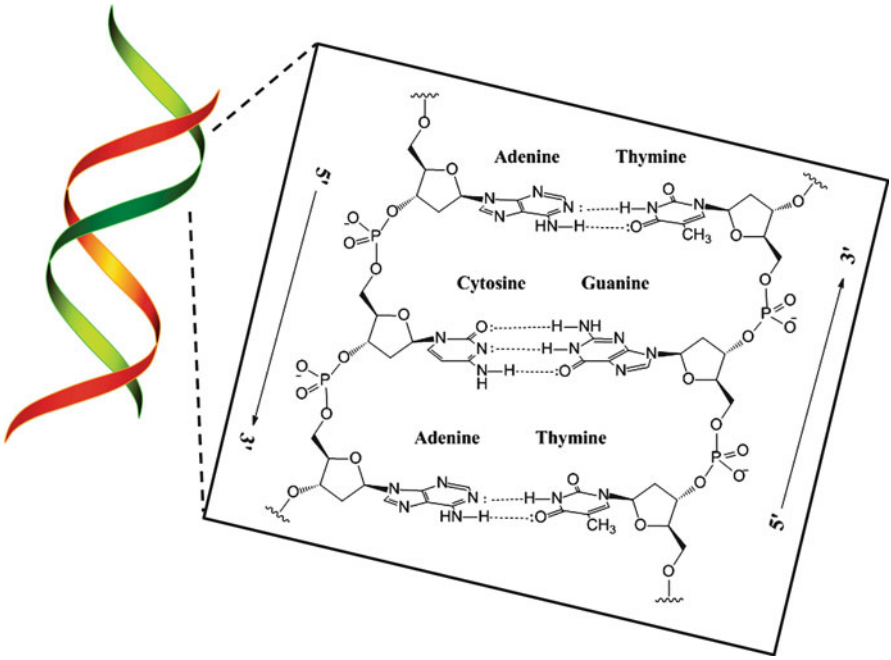

Fig. 1.1 Schematic of a DNA double helix. The two antiparallel strands of a DNA double helix are shown as *green* and *red ribbons*. The detailed positioning of the phosphate backbone, ribose sugar, and nitrogenous bases as well as the hydrogen bonding network is shown in the magnified inset

storage of genetic information. One of the first well-reasoned theoretical predictions that stimulated the search for genetic molecule can be attributed to the ideas introduced by Erwin Schrödinger in his popular book *What Is life* in 1944. Schrödinger proposed that the chromosomes, which were known at that time to be the information carriers, are the "aperiodic crystals" where covalent chemical bonds are utilized to enhance the genetic information. The search for the genetic molecule culminated in 1953 with the discovery of DNA structure by James Watson and Francis Crick [1]. In 1962, Watson, Crick, and Maurice Wilkins were awarded Nobel Prize in Physiology and Medicine for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material. This information is written in a code composed of a four-letter alphabet system which must be duplicated accurately as cells divide in order to maintain genomic integrity. DNA is a polymer of nucleotides, where each building block consists of a phosphate group, a five-carbon sugar (deoxyribose), and one of four nitrogenous bases (*A*denine, *G*uanine, *C*ytosine, and *T*hymine).

The structure of DNA proposed in 1953 by James Watson and Francis Crick, and also Rosalind Franklin and Maurice Wilkins, has been paramount in facilitating nucleic acid research. The structure revealed that two strands of DNA intertwine to form a right-handed "double helix" (Fig. 1.1). The backbone of the helix is formed by alternating units of the phosphate and carbon groups while the nitrogenous bases point

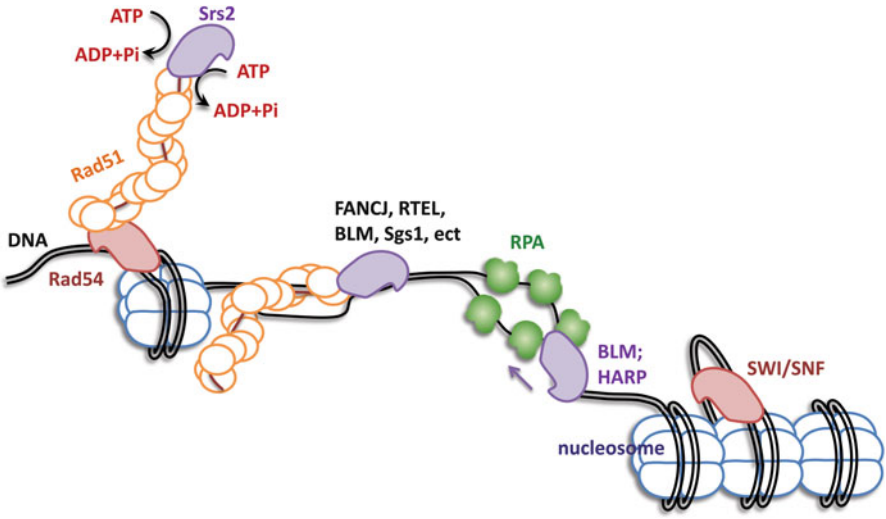

Fig. 1.2 DNA helicases, helicase-like motors, and their diverse substrates. Eukaryotic genomic DNA is organized in a chromatin, where an approximately 146 bp of DNA is wrapped around each histone octamer (*blue*), which consists of two copies of H2A, H2B, H3, and H4 histone proteins. Adjacent nucleosome core particles are tethered together by linker DNA and forms a "beads on a string" structure. Examples of other important DNA replication, recombination, and repair machinery are also shown and include SWI/SNF family chromatin remodeler, which repositions nucleosomes and enables the access to genetic information to the transcription and repair systems, DNA annealing helicases BLM and HARP, anti-recombinases Srs2, FANCJ, RTEL, BLM and Sgs1, and the Rad54 protein, which has both pro-recombinogenic and chromatin remodeling functions

towards the center of the helical axis. The sequential arrangement of these bases is how genetic information is written and stored. Only specific pairs of bases can properly bond together between the two DNA strands without perturbing the double-stranded helix (adenine with thymine and cytosine with guanine); hence, if the base sequence of one DNA chain is known, then the identity of bases on the other strand can be determined empirically. The double helix structure of DNA and the hydrogen bonding patterns of the nitrogenous bases have provided invaluable insight into how the genetic code is replicated—the two strands of the double helix must be first separated and then the replication machinery can read the sequence of the template bases and incorporate the corresponding partner bases to regenerate the DNA duplex. Although this is simple conceptually, DNA replication is highly regulated in order to maintain genomic stability. During semiconservative replication [2], the complete DNA content of the cell has to be duplicated in a timely manner and exactly once.

In addition, DNA is a large macromolecule and the human genome, which is on the order of ~3,000 million base pairs in length and arranged into 23 distinct chromosomes. To cope with its enormous length, genomic DNA is tightly packaged and stored, an arrangement that allows the control of the accessibility of this information.

DNA in each chromosome is packaged as protein–DNA complexes called chromatin, and the basic structural unit of chromatin is a nucleosome [3] (Fig. 1.2) (see Chap. 13). One purpose of these complex arrangements is to package the vast

amount of genetic material (almost 2 m in length) within the nucleus inside which space is limited (roughly 10 μm in diameter). To do so, the DNA is wrapped around a core of histone proteins in each nucleosome, much like how thread is spun around a spindle (Fig. 1.2). Histones comprise the majority of protein found in chromatin, and ~146 bp of DNA can be wrapped around each histone core in the nucleosome. These tightly arranged protein–DNA complexes condense to form chromatin and are further organized into chromosomes. As described above, access to genetic information is tightly controlled and genetic DNA is protected and stored as chromatin. These protein–DNA complexes must be "remodeled" in order to gain access to the genetic code. Chromatin assembly and remodeling will be discussed in greater detail in Chap. 13.

Cellular DNA undergoes harmful modifications every day as a result of exposure to UV light, environmental stress, and toxic chemicals. DNA damage can also result from errors during DNA synthesis. Damaged DNA must be repaired promptly and efficiently; otherwise, the replication machinery can incorporate the wrong nitrogenous base, leave nicks and gaps, and stall or disengage during subsequent rounds of DNA synthesis, resulting in deleterious mutations and chromosomal instability. The cell utilizes a number of repair pathways to prevent the loss of genetic information. The enzymes that are involved in the repair process are specific to the type of DNA damage encountered and depend on the stage of the cell cycle. Not surprisingly, defects in key components of these systems in humans are associated with a broad spectrum of disorders, usually characterized by premature aging, susceptibility to cancers, and other diseases bearing hallmarks of aging, immunodeficiency, or mental retardation (see Chap. 6). Similar to DNA replication, in order for the DNA repair machinery to gain access to the genetic code, the two strands of the double helix must first be "unwound." In some cases, because DNA is tightly packaged in chromatin, these protein–DNA complexes need to be restructured to expose the DNA region of interest. The enzymes which couple chemical energy to unwind the DNA duplex are commonly referred to as helicases. Related motors also work as chromatin remodelers, which restructure chromosome organization and thereby enabling or restricting access to DNA.

DNA Helicases: Ubiquitous Molecular Motors

The first DNA helicase, *Escherichia coli* TraI (helicase I), was purified and characterized in 1976 [4, 5]. As more helicases were identified and reported in the literature, helicase "signature motifs" were identified [6]. These highly conserved amino acid domains are involved in the binding and hydrolysis of nucleoside triphosphate (NTP), the energy source required to separate the stable double-stranded DNA (dsDNA). So far hundreds of helicases have been identified across different organisms. It is estimated that approximately 1% of the prokaryotic and eukaryotic genomes encode for proteins containing helicase signature motifs. Helicases have been classified into superfamilies (SF) and families based on amino acid patterns and the presence of the

helicase motifs, with a majority of enzymes falling into SF1 and SF2 [6]. However, this original classification scheme was developed when biochemical and structural data were limited; therefore, bioinformatics-based analysis and nomenclature often fail to provide a reliable mechanistic insight into how a helicase might function. Recently, a new classification approach has been proposed to differentiate between enzymes that move along single-stranded DNA (ssDNA) (type A) and dsDNA (type B) with either 3′–5′ (type α) or 5′–3′ (type β) directionality [7]. This book will mainly focus on discussing the features and characteristics of DNA helicases although we note that there are many enzymes that act on RNA duplexes and RNA–DNA hybrids. The properties of these RNA helicases and their role in RNA metabolism and diseases are well reviewed in the following literature [8, 9].

Mechanistic Considerations

The DNA double helix is thermodynamically stable under normal physiological conditions. Therefore, energy input is required to destabilize the duplex. Helicase activity is defined as the ability to use energy from nucleotide binding and hydrolysis to separate dsDNA into ssDNA. This unique activity is quite distinct, at least at a first glance, from duplex melting by the ssDNA binding proteins, which can destabilize short DNA duplexes transiently simply because they bind and capture ssDNA with high affinity and selectivity. A processive helicase is an enzyme that unwinds many base pairs per binding event. In contrast, a distributive helicase unwinds only a limited region of dsDNA before dissociating and typically separates the duplex by virtue of binding to it akin to the ssDNA binding proteins that destabilize duplexes exclusively at the expense of the potential energy. An important distinction, however, is that the distributive helicases still require ATP hydrolysis, which supplies the free energy to recycle the helicase and reset it for the next binding/melting event. In order for a helicase to unwind DNA processively, it must also be able to move along the DNA filament (i.e., to translocate) and couple this directional motion along the DNA lattice to strand separation activity. While processive helicase activity certainly requires processive translocation, the reverse is not necessarily true—a protein that translocates along DNA processively may not have robust helicase activity; therefore, it is more appropriate to refer to such enzymes as translocases. Examples of such motors include the *E. coli* Rep and UvrD enzymes as well as *Bacillus stearothermophlius* PcrA [10–13], which are reviewed in Chap. 2 as well as dsDNA translocases reviewed in Chaps. 12 and 13. Since helicases are identified and classified based on the presence of conserved signature motifs, it is not known whether a helicase would have bona fide strand separation activity or translocase activity solely based on amino acid patterns. In fact, it is now evident that there are some enzymes that are classified as helicases but function instead as molecular switches and remodel protein–DNA complexes without moving along nucleic acid, while others act on substrates that are not nucleic acids at all [14–16]. Hence, the presence of these conserved helicase motifs only designates these enzymes as

nucleic acid-stimulated NTPases, and their exact function(s) must be examined experimentally.

Processive DNA unwinding requires a helicase to undergo a series of repeated "steps" along the DNA lattice until the duplex is fully unwound [17, 18]. Each step involves a number of processes such as NTP binding, hydrolysis, phosphate release, base pair melting or capturing of the spontaneously melted bases, and translocation. The physical step size of a helicase is defined as the mean distance change of the center of mass of the enzyme in between two repeated rounds of DNA unwinding or per NTP molecule hydrolyzed. This is a useful and informative parameter because it not only gives a helicase a physical characteristic but also provides a mechanistic constraint for its function. However, it is not always possible to measure the physical step size directly since high resolution techniques and a suitable helicase model system are both required. Using high precision single-molecule approaches, the kinesin motor has been shown to take discrete 8 nm steps along microtubules [19]. Similar optical trapping methods have revealed that the Hepatitis C virus (HCV) NS3 RNA helicase takes large steps of about 11 bp and that DNA unwinding occurs in smaller ~1 bp substeps [20]. Alternatively, DNA unwinding can also be expressed as a multistep process involving the cycling of a series of repeated rate-limiting steps. Since it is not known a priori which physical process is rate limiting in the unwinding mechanism (e.g., NTP binding, base pair melting, translocation), the kinetic step size refers to the average movement of the enzyme in between two successive rate-limiting steps of the reaction. While they are conceptually similar, the kinetic step size could differ from the physical step size [21]. For example, a helicase can unwind DNA in discrete physical steps of 1 bp per step but may need to undergo a slow step after every X base pairs are unwound as a result of molecular rearrangement, phosphate release, etc. In this case, the observed kinetic step size will be X bp even though the actual physical step size is 1 bp.

Another important parameter is the stepping rate, which is defined as the number of steps a helicase takes per second. Depending on the helicase system and the type of experiment, the stepping rate and step size could be measured for DNA unwinding and/or translocation. The product of the step size and the stepping rate is the macroscopic rate of unwinding or translocation and has units of bp (or nt) per second. In the extreme case, the *E. coli* TraI and RecBCD helicases can unwind dsDNA with high processivity and with rates of over 1,000 bp/s [22–24]. In contrast, the *E. coli* RepΔ2B monomer only unwinds dsDNA with a rate of 226 ± 28 bp/s and limited processivity [10]. It is important to note that translocase and helicase activities are strongly sensitive to solution conditions (temperature, pH, salt concentration, salt type, NTP concentration, etc.), and one must keep this in mind when making quantitative comparisons between different studies.

Mechanistic models by which helicases unwind and translocate along DNA will be discussed in more detail in the subsequent chapters. Two limiting mechanisms have been suggested for DNA unwinding—active and passive [17, 25–28]. A passive helicase simply translocates along ssDNA with a directional bias. When it encounters a ds/ssDNA junction, the enzyme is stopped and must wait for the duplex to open through transient thermal fluctuations. Exploiting this phenomenon, a passive helicase

will eventually advance into the partially open duplex using its translocase activity, capture the spontaneously open fork, and prevent the two ssDNA strands from reannealing. In contrast, an active helicase directly interacts and destabilizes the DNA duplex and lowers the free energy of the fork. Therefore, the ssDNA translocation rate of a passive enzyme is predicted to be much faster than its unwinding rate since it has to wait for the DNA duplex to open transiently. In contrast the active unwinding model predicts this enzyme will have an unwinding rate similar to its rate of ssDNA translocation. The terms active and passive simply define the two extremes of a scale and there is an entire spectrum of possibilities in between. Under solution conditions similar to that of *E. coli* (pH 7.0, 3 mM Mg^{2+}, 0.2 M K^{+}, 8 mM ATP, 1 mM ADP, 8 mM P_i), ΔG_{ATP} is ~−13 kcal mol^{-1} at 25 °C while $\Delta G_{base\ pair}$ is ~1–1.5 kcal mol^{-1}. Therefore, if the coupling of ATP hydrolysis to DNA unwinding is 100% efficient, ~9–12 bp can be melted per molecule of ATP hydrolyzed [17]. Efforts have been made to quantify the "activeness" of a helicase based on its unwinding and translocation rates and to estimate NTP consumption during these processes [27–29].

There are several models with which the duplex strand separation is described. In the *E. coli* RecBCD enzyme, a "separation pin" in the RecC subunit was proposed to function as a molecular wedge [30]. This aromatic amino acid forms base stacking interactions with the DNA duplex, and base pair melting is achieved as a result of the RecB (a 3′–5′ helicase) and RecD (a 5′–3′ helicase) motors translocating along two strands of DNA. However, RecBCD is able to use its binding free energy to melt out 5–6 bp of DNA upon binding to a DNA end, and the RecB monomer, devoid of the separation pin, is able to unwind short DNA duplexes [31, 32]. The RecB subunit also has an "arm" domain which forms contact with the duplex ahead of the ds/ss junction. Taken together, RecBCD may be able to directly interact with the duplex and destabilize the fork [33]. In the case of ring-shaped hexameric helicases (i.e., DnaB, T7), strand exclusion models have been proposed in which the hexameric ring encircles one strand of the DNA duplex via a single-stranded loading region [34–36] (see also Chap. 5). Upon strand separation, the hexamer advances into the fork and the opposite strand is sterically excluded from the ring. Notably, various "pin" and "wedge" structures have been implicated in the dsDNA unwinding by virtually every non-hexameric helicase independent on where it lies of the passiveness continuum.

Brownian Ratchet vs. Powerstroke

Two distinct mechanisms utilized by helicases to ensure directional translocation have been considered: Brownian ratchet and powerstroke. A Brownian motor (thermal ratchet) is driven by thermal fluctuations. Directional movement of this "ratchet" is achieved as a result of an anisotropic energy potential across the DNA lattice although the nature and the origin of this asymmetry is not well understood and is likely to vary in different enzymes. However, in such a model system, the helicase only needs to switch between two conformational states (i.e., high and low affinity for DNA) which could be triggered by NTP binding and hydrolysis, and the helicase

will travel along the path of the anisotropic potential akin to guided diffusion. This model requires simply one DNA binding site on the helicase, and predicts low translocation and unwinding processivity since NTP hydrolysis is loosely coupled to movement.

In contrast, a powerstroke motor or stepping motor requires at least two DNA binding sites on the helicase. The best example of this is a molecular "inch worm" mechanism described for several SF1 helicases [37] in which translocation is driven by movements of the 1A and 2A subdomains. Nucleotide is bound in a cleft between these two RecA-like motifs and the state of the cofactor leads to a series of conformational changes that move the two subdomains closer together. In such an inch worm mechanism, the leading domain is always in the front and similarly the trailing domain always follows behind. Directionality is achieved in the initiation complex as a result of binding orientation [38, 39]. In contract, in a hand-over-hand stepping model like in the kinesin family motors, movement is similar to walking where the leading domain becomes the trailing domain in the next cycle and vice versa [40].

Structures of DNA Helicases

There are a number of high resolution structures of DNA helicases either in the apo form or bound to a DNA substrate with (or without) a nucleotide cofactor. A common structural feature is the motor core domain, which adopts two RecA-like folds (Fig. 1.3). Chapters 2 and 3 will discuss in detail the structural features as well as mechanisms of SF1 and SF2 helicases, respectively. The canonical motor core consists of two globular subdomains 1A and 2A, and the nucleotide cofactor binds in a cleft in between these two regions. Although the motor core is conserved amongst helicases, their diversity stems from a number of factors such as oligomeric state, presence of accessory and regulatory domains, and DNA substrate specificity. For example, the XPD helicase, which is an SF2Aβ enzyme, possesses two auxiliary domains in addition to the canonical helicase motor core: an Arch domain and an iron-sulfur cluster (FeS) domain. Both of these regions are necessary for unwinding activity and may be involved in stabilizing the interaction between XPD and the ds/ssDNA junction [38, 41]. In *E. coli* Rep, an SF1Aα enzyme, the presence of a 2B subdomain has an auto-inhibitory effect which may play a regulatory role in its translocation and unwinding activities [10]. Rep monomers can translocate rapidly and processively along ssDNA in vitro but are unable to unwind even short DNA duplexes. Rep helicase activity can be stimulated by removing the 2B subdomain artificially, thus forming the RepΔ2B monomer, or the wild-type enzyme can undergo dimerization to promote DNA unwinding. The presence of modular accessory domains can provide an extra level of control and regulation of helicase and translocase activities in vivo, and in some systems these accessory domains can target the enzymes to specific DNA structures, or they can be scaffolds for forming higher ordered complexes with mediator proteins. Helicases do indeed come in all

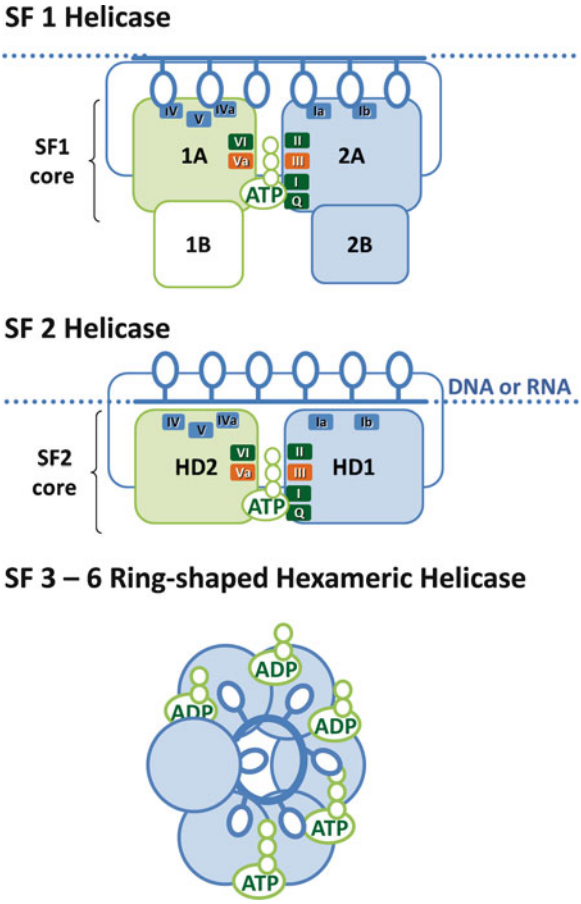

Fig. 1.3 Cartoon representations of DNA helicases from different superfamilies. Schematics of an SF1, SF2, and ringed-shaped hexameric helicase are shown. For SF1 helicases, the subdomains are as indicated, and the 1A and 2A subdomains form a motor core and adopt a "RecA"-like fold. The same is true for HD1 and HD2 in SF2 helicases. Nucleotide cofactor is bound in a cleft between these two subdomains. In a ring-shaped hexameric helicase, the hexamer encircles the DNA in the center pore and one strand of the nucleic acid is excluded from the helicase as it unwinds the duplex. Nucleotide cofactor is bound at the subunit interfaces. Because there are six potential nucleotide binding sites, these sites can have different nucleotide cofactor states (ATP bound, ADP, empty, etc.) depending on whether the helicases uses a random or ordered mechanism for ATP binding and hydrolysis

shapes and sizes. Some are monomeric while others function as homodimers or heterodimers. Other enzymes form ring-shaped hexamers (Fig. 1.3 and Chap. 4), and some are integral parts of complex transcription and replication machinery.

Helicases can modulate their activities not only through protein–protein interactions but also through posttranslational modifications (PTMs) such as phosphorylation, ubiquitylation, and sumoylation. Modified helicases can possess intrinsically different properties (i.e., unwinding and translocation activities), and PTMs can enable interactions with other protein partners, and target the helicase to certain sites for function. For example, the FANCJ helicase, upon phosphorylation on S990, enables interaction with the BRCA1 tumor suppressor protein [42, 43]. How this interaction influences FANCJ unwinding, translocation, and its ability to disrupt holiday junctions and G-quadruplexes is still not well understood. Since many mammalian helicases are subject to PTMs, it has become increasingly important to understand the functional roles of these modifications as an additional component to mechanistic studies of DNA helicases.

Assays for Helicase Activity

When helicases were first identified, DNA unwinding activity was inferred by coupling the strand separation reaction with nuclease digestion [44]. If a helicase unwinds dsDNA in the presence of NTP, then the newly generated ssDNA will be sensitive to nuclease degradation, and the cleavage products can be analyzed by scintillation counting. A more direct and the most widely used approach is a strand displacement experiment in which one strand of the DNA duplex is either radiolabeled with ^{32}P or fluorescently labeled with a fluorophore so that this strand can be detected when the duplex is unwound and the ssDNA is displaced into solution [45, 46]. Since the labeled reporter strand can hybridize with the other unlabeled unwound ssDNA product to form the original duplex, excess unlabeled ssDNA can be added to capture this other strand and thereby preventing reannealing. Strand displacement assays can be either discontinuous or continuous [21]. In a discontinuous assay, an initiation complex is formed between the helicase and duplex DNA substrate in the absence of nucleotide cofactor. DNA unwinding is then initiated with the addition of NTP and an unlabeled ssDNA trap. The reaction is quenched in a series of predetermined time intervals and the products from each time point can be separated by gel electrophoresis and analyzed using autoradiography. This type of a gel-based assay is "all or none" because at any given time point only the fully unwound product and the original duplex can be detected. Any partially unwound intermediates will "re-zip" and form the fully base-paired duplex when the reaction is quenched. However, as discussed later below, information regarding these transient intermediate species can be extracted from such an all or none experiment. In a continuous assay, DNA unwinding is monitored in real time continuously. Each of these approaches has distinct advantages and limitations. A discontinuous assay allows one to analyze the reaction products at each time point and thus the extent of DNA unwinding can be directly measured. Also, the gel-based assay can resolve different types of products that are formed. This is especially important when a helicase acts on a complex multi-strand substrate (e.g., replication fork, holiday junction, four-way junction), since one can unambiguously define its substrate preference by separating and directly observing the unwinding products. However, due to the discontinuous nature of the experiment, the number of data points one can collect within a reasonable amount of time is limited. Therefore, it is difficult to construct a detailed time course and to examine DNA unwinding as a function of different reaction conditions.

In contrast, in a continuous unwinding experiment, many data points can be obtained for a given time course, and different DNA substrates and solution conditions can be examined readily. However, a continuous unwinding experiment typically requires a difference in spectroscopic signal coming from either the protein or the DNA molecule when the DNA is unwound; hence, the relationship between this signal change and unwound DNA products need to be determined and calibrated independently in order to determine the extent of DNA unwinding. While all gel-based discontinuous assays are "all or none," continuous unwinding assays can be either depending on how the DNA substrate is constructed and how DNA unwinding

is monitored. Analogous all or none experiments can be performed in a continuous assay in which a pair of fluorescent dyes can be placed across the DNA duplex on the two strands. These two fluorophores can undergo Forster Resonance Energy Transfer (FRET) in the duplex since they are in close proximity. Because FRET efficiency is initially high, the donor fluorophore is quenched by the presence of the acceptor fluorophore as a result of non-radiative transfer of energy; however, when the two strands are unwound, separated, and displaced into solution, FRET efficiency decreases and the quantum yield of the donor fluorophore increases. In the case of RAD3 family helicases (e.g., XPD, FANCJ, RTEL), which possess an innate FeS cluster, this domain quenches fluorescence as a function of distance and therefore can be used to monitor either DNA unwinding or ssDNA translocation. Variations to continuous ensemble unwinding experiments include coupling the unwinding reaction with the binding of fluorescently labeled *E. coli* single-stranded binding protein (SSB), which undergoes a change in fluorescence intensity when it binds to ssDNA rapidly and with high affinity [47]. Alternatively, fluorescent dyes which intercalate between the nitrogenous bases can also be used to monitor DNA unwinding since they are either displaced upon strand separation or have different fluorescence properties when bound to ssDNA vs. dsDNA [48]. Since intercalating agents can have adverse effects on helicase activity, gel-based unwinding experiments should be carried out as an important control experiment. Continuous and discontinuous assays have different strengths and limitations, and they complement each other in the type of information each provides. Fluorescence-based continuous methods give better estimates of kinetic parameters since many data points can be collected for a given time course while gel-based discontinuous methods give better insight into the extent of DNA unwinding and the types of products that are formed.

The average kinetic step size of a helicase can be estimated using either continuous or discontinuous "all or none" strand displacement methods. As discussed above, in such an experiment, only the fully unwound DNA duplex is detected. Since helicase-catalyzed DNA unwinding is a multistep process in which the helicase undergoes a series of repeated steps along the DNA substrate until it is unwound and because only the fully unwound DNA product is monitored in an "all or none" experiment, a lag in product formation is observed in the beginning of the unwinding time course. The duration of this lag phase is proportional to the number of steps the helicase must take to fully unwind the DNA and therefore it is sensitive to the duplex length. By repeating the unwinding experiment as a function of increasing duplex length, the longer DNA substrates will exhibit longer lag phases in the unwinding time courses and these lag kinetics can be globally analyzed to estimate the average kinetic step size for DNA unwinding [21, 49].

With the recent advances and developments of instrumentation, there are now several "single-molecule" techniques with which a single DNA or a single helicase molecule can be observed. Several distinct single-molecule unwinding assays have been used to study many DNA helicases [50]. In total internal reflection fluorescence (TIRF) microscopy, DNA or helicase molecules are tethered onto a microscope slide surface and either moiety can be fluorescently labeled. By using low concentrations of materials as well as evanescent wave excitation, the unwinding or translocation

activities of individual helicase molecules can be observed by using FRET or protein-induced fluorescence enhancement (or quenching) as a metric of distance. Alternatively, conformation dynamics of helicase–DNA interactions or inter-domain movements can also be examined.

Another single-molecule approach is the use of high precision optical trap(s), and there are several variations of instrumentation used in these experiments. One example is the use of dual optical traps in which a DNA substrate has two handles, typically polystyrene beads, that are each held in place by the laser traps [51]. The forces exerted on the beads as well as their relative positions to the center of each trap can be measured and detected with high precision and accuracy. The DNA substrate held by the "optical tweezer" can be pulled and stretched by moving the position of one of the traps. Typically, a force vs. ssDNA extension curve is experimentally determined independently in order to convert the measured forces in an unwinding experiment to the number of bases unwound. If a DNA hairpin is held via the two handles on the optical traps, then as a helicase unwinds the hairpin substrate, 2 nts are released per base pair unwound. Therefore, the extent of DNA unwinding is calculated from the calibration curve after the relationship between force and extension (position) is established. Under high forces (>12 pN), force vs. ssDNA extension can be modeled using a freely jointed chain model. Under very lower forces (2 pN), secondary structure in the ssDNA may form and the worm-like chain model would be a poor representation of force vs. extension [52]. Despite this ambiguous representation, the compactness of ssDNA at low applied forces provides a second regime where ssDNA products of the helicase-catalyzed unwinding can be distinguished from the duplex substrate [27].

Alternatively, ssDNA translocation can be examined by using a duplex DNA substrate with a nick, and ssDNA can be generated by "pulling" on one of the handles thereby ripping open a region of the duplex mechanically. The helicase then be able to bind to the newly generated ssDNA region and translocate along the tract until it reaches the duplex upon which DNA unwinding occurs and the force exerted on the polystyrene bead changes. At this point another region of ssDNA can be generated mechanically again to observe another round of translocation. Although ssDNA translocation is not monitored directly in such an experiment, translocation activity can be inferred by determining the length of the ssDNA region opened mechanically, and the time it takes before unwinding is detected. These types of experiments have been used to examine the translocation and unwinding activities of the bacteriophage T7 helicase and also the HCV NS3 RNA helicase. One advantage of these optical tweezer experiments is that forward and reverse motions, and also pausing events, can be detected [53–55]. Since these events are stochastic in nature, it is not possible to detect them using traditional ensemble methods described above. In addition, DNA unwinding and ssDNA translocation can be examined in a single experiment under the same conditions [27], which enables one to compare these two activities and determine whether the helicase functions as an active or passive motor.

Another type of optical trap experiment employs the use of a single optical trap. A long piece of DNA (e.g., bacteriophage λ) is held by the trap on one end via a polystyrene bead. Instead of holding the DNA at another end, flow is applied to the system

so that the DNA is stretched in the direction of flow. A DNA intercalating dye (e.g., YOYO-1) is used to visualize the nucleic acid. When a helicase binds and unwinds the DNA, the dye is displaced and a loss of fluorescence signal is observed. *E. coli* RecBCD has been shown using such an experiment to pause at Chi regulatory DNA sequences (5′-GCTGGTGG-3′) [22, 24, 54]. Building upon this concept, a series of hundreds of trapped DNA molecules can be lined up and stretched, thereby forming a "DNA curtain" [56, 57]. This method allows one to examine the activities of many more helicase molecules simultaneously, and RecBCD has been shown to disrupt RNA polymerase, lac repressor, and other protein–DNA complexes including nucleosomes [58].

Helicases in Diseases

The genetic code is written as the sequences of the nitrogenous bases in DNA. This information is stored within duplex DNA, which is thermodynamically stable. In order to access the genetic information, dsDNA must first be unwound; hence, helicase activity is required during DNA replication, recombination, and repair. Mutations in human helicases such as BLM, WRN, and XPD can result in Bloom's syndrome, Werner's syndrome, and Xeroderma pigmentosum, respectively. These diseases are characterized by signs of premature aging as a result of genetic instability. Defects in the FANCJ helicase is linked to Fanconi anemia, a chromosomal instability disorder which results in bone marrow failure and susceptibility to breast cancer, ovarian cancer, and acute myeloid leukemia. The molecular basis and mechanisms by which the malfunctions of these enzymes result in diseases remain unclear. This is because helicases are involved in an intricate network of DNA metabolic enzymes and mediator proteins, and it is not known specifically which interactions are functionally important. In Chap. 6, DNA helicases associated with cancer, aging, and other genetic instability disorders will be discussed in detail; potential drug targets and therapeutics will also be described.

Remaining Questions

Although the field of DNA helicases research has matured immensely over the last decade or so, a number of gaps in our understanding of structure functional relationships, regulation, and integration of DNA helicases remain wide open. Recent advancements in instrumentation and method development have enabled researchers to monitor DNA unwinding at a single-molecule level with up to one base pair resolution. We are now poised to better address some fundamental questions of DNA helicases—how the NTP binding and hydrolysis are coupled to base pair melting and translocation, and how do helicases unwind and move along DNA? There are a number of detailed biochemical and structural studies using bacterial helicases as model systems in effort to address these questions. However, mechanistic

studies of mammalian helicases and our understanding of these enzymes are limited to this date. One research challenge is that these mammalian enzymes are typically more complex since they may possess multiple motor subunits or may be a part of complicated replication and recombination machinery. Furthermore, these helicases are often regulated by posttranslational modifications (PTMs) or interaction with regulatory factors. As a result, it has been difficult to reconstitute these systems for traditional bulk phase studies in vitro. One way to overcome this obstacle is to bypass this problem altogether. For example, using powerful single-molecule methods, it is possible to tether the entire cellular pool of a helicase of interest on a microscope slide. Enzymes possessing PTMs and the PTM type can be identified on the slide surface using fluorescence probes specific for the PTM. This enables all positions of modified and unmodified enzymes to be identified and then subsequent studies of helicase activity or translocase activity can be correlated to the PTMs. Powerful microscopy approaches have also been used to visualize movements of helicase subdomains. Continual effort is made to correlate these movements to DNA binding as well as nucleotide binding/hydrolysis. These types of experiments combined with classical mechanistic studies will provide a better understanding of how helicases harness chemical energy to do mechanical work, and how defects of helicase function are associated with genetic diseases.

Acknowledgements We are gratefully acknowledge support by the American Cancer Society (RSG-09-182-01-DMC to MS and PF-11-243-01-DMC to CGW) and Howard Hughes Medical Institute (Early Career Scientist Award).

References

1. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature. 1953;171(4356):737–8.
2. Meselson M, Stahl FW. The replication of DNA in Escherichia coli. Proc Natl Acad Sci U S A. 1958;44(7):671–82.
3. Andrews AJ, Luger K. Nucleosome structure(s) and stability: variations on a theme. Annu Rev Biophys. 2011;40:99–117.
4. Abdel-Monem M, Durwald H, Hoffmann-Berling H. Enzymic unwinding of DNA. 2. Chain separation by an ATP-dependent DNA unwinding enzyme. Eur J Biochem. 1976;65(2):441–9.
5. Abdel-Monem M, Hoffmann-Berling H. Enzymatic unwinding of DNA. 1. Purification and characterization of a DNA-dependent ATPase from Escherichia coli. Eur J Biochem. 1976;65(2):431–40.
6. Gorbalenya AE, Koonin EV. Helicases: amino acid sequence comparisons and structure-function relationships. Curr Opin Struct Biol. 1993;3:419–29.
7. Singleton MR, Dillingham MS, Wigley DB. Structure and mechanism of helicases and nucleic acid translocases. Annu Rev Biochem. 2007;76:23–50.
8. Jankowsky E, Fairman ME. RNA helicases—one fold for many functions. Curr Opin Struct Biol. 2007;17(3):316–24.
9. Jankowsky E, Fairman ME, Yang Q. RNA helicases: versatile ATP-driven nanomotors. J Nanosci Nanotechnol. 2005;5(12):1983–9.
10. Brendza KM, et al. Autoinhibition of *Escherichia coli* Rep monomer helicase activity by its 2B subdomain. Proc Natl Acad Sci U S A. 2005;102(29):10076–81.

11. Maluf NK, Fischer CJ, Lohman TM. A dimer of *Escherichia coli* UvrD is the active form of the helicase in vitro. J Mol Biol. 2003;325(5):913–35.
12. Niedziela-Majka A, et al. Bacillus stearothermophilus PcrA monomer is a single-stranded DNA translocase but not a processive helicase in vitro. J Biol Chem. 2007;282(37): 27076–85.
13. Tomko EJ, et al. A nonuniform stepping mechanism for E. coli UvrD monomer translocation along single-stranded DNA. Mol Cell. 2007;26(3):335–47.
14. Park SK, et al. RNA helicase activity of *Escherichia coli* SecA protein. Biochem Biophys Res Commun. 1997;235:593–7.
15. Gelis I, et al. Structural basis for signal-sequence recognition by the translocase motor SecA as determined by NMR. Cell. 2007;131(4):756–69.
16. Martin A, Baker TA, Sauer RT. Rebuilt AAA+ motors reveal operating principles for ATP-fuelled machines. Nature. 2005;437(7062):1115–20.
17. Lohman TM, Bjornson KP. Mechanisms of helicase-catalyzed DNA unwinding. Annu Rev Biochem. 1996;65:169–214.
18. Patel SS, Donmez I. Mechanisms of helicases. J Biol Chem. 2006;281(27):18265–8.
19. Schnitzer MJ, Block SM. Kinesin hydrolyses one ATP per 8-nm step. Nature. 1997;388: 386–90.
20. Cheng W, et al. Single-base pair unwinding and asynchronous RNA release by the hepatitis C virus NS3 helicase. Science. 2011;333(6050):1746–9.
21. Lucius AL, et al. General methods for analysis of sequential "n-step" kinetic mechanisms: application to single turnover kinetics of helicase-catalyzed DNA unwinding. Biophys J. 2003;85(4):2224–39.
22. Bianco PR, et al. Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. Nature. 2001;409:374–8.
23. Sikora B, et al. DNA unwinding by Escherichia coli DNA helicase I (TraI) provides evidence for a processive monomeric molecular motor. J Biol Chem. 2006;281(47):36110–6.
24. Spies M, et al. RecBCD enzyme switches lead motor subunits in response to chi recognition. Cell. 2007;131(4):694–705.
25. Betterton MD, Julicher F. Opening of nucleic-acid double strands by helicases: active versus passive opening. Phys Rev E Stat Nonlin Soft Matter Phys. 2005;71(1):011904.
26. Delagoutte E, von Hippel PH. Helicase mechanisms and the coupling of helicases within macromolecular machines. Part I: structures and properties of isolated helicases. Q Rev Biophys. 2002;35(4):431–78.
27. Johnson DS, et al. Single-molecule studies reveal dynamics of DNA unwinding by the ring-shaped T7 helicase. Cell. 2007;129(7):1299–309.
28. Manosas M, et al. Active and passive mechanisms of helicases. Nucleic Acids Res. 2010;38(16):5518–26.
29. Byrd AK, et al. Dda helicase tightly couples translocation on single-stranded DNA to unwinding of duplex DNA: Dda is an optimally active helicase. J Mol Biol. 2012;420(3):141–54.
30. Singleton MR, et al. Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks. Nature. 2004;432(7014):187–93.
31. Farah JA, Smith GR. The RecBCD enzyme initiation complex for DNA unwinding: enzyme positioning and DNA opening. J Mol Biol. 1997;272:699–715.
32. Wong CJ, Lucius AL, Lohman TM. Energetics of DNA end binding by *E. coli* RecBC and RecBCD helicases indicate loop formation in the 3′-single-stranded DNA tail. J Mol Biol. 2005;352(4):765–82.
33. Wu CG, Bradford C, Lohman TM. Escherichia coli RecBC helicase has two translocase activities controlled by a single ATPase motor. Nat Struct Mol Biol. 2010;17(10):1210–7.
34. Ahnert P, Patel SS. Asymmetric interactions of hexameric bacteriophage T7 DNA helicase with the 5′- and 3′-tails of the forked DNA substrate. J Biol Chem. 1997;272(51):32267–73.
35. Hacker KJ, Johnson KA. A hexameric helicase encircles one DNA strand and excludes the other during DNA unwinding. Biochemistry. 1997;36:14080–7.

36. Galletto R, Jezewska MJ, Bujalowski W. Unzipping mechanism of the double-stranded DNA unwinding by a hexameric helicase: the effect of the 3′ arm and the stability of the dsDNA on the unwinding activity of the *Escherichia coli* DnaB helicase. J Mol Biol. 2004;343(1):101–14.
37. Eoff RL, Raney KD. Helicase-catalysed translocation and strand separation. Biochem Soc Trans. 2005;33(Pt 6):1474–8.
38. Pugh RA, Wu CG, Spies M. Regulation of translocation polarity by helicase domain 1 in SF2B helicases. EMBO J. 2012;31(2):503–14.
39. Saikrishnan K, et al. DNA binding to RecD: role of the 1B domain in SF1B helicase activity. EMBO J. 2008;27(16):2222–9.
40. Tomishige M, Stuurman N, Vale RD. Single-molecule observations of neck linker conformational changes in the kinesin motor protein. Nat Struct Mol Biol. 2006;13(10):887–94.
41. Kuper J, et al. Functional and structural studies of the nucleotide excision repair helicase XPD suggest a polarity for DNA translocation. EMBO J. 2012;31(2):494–502.
42. Cantor SB, et al. BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell. 2001;105(1):149–60.
43. Clapperton JA, et al. Structure and mechanism of BRCA1 BRCT domain recognition of phosphorylated BACH1 with implications for cancer. Nat Struct Mol Biol. 2004;11(6):512–8.
44. Kuhn B, Abdel-Monem M, Hoffmann-Berling H. DNA helicases. Cold Spring Harb Symp Quant Biol. 1979;43(Pt 1):63–7.
45. Venkatesan M, Silver LL, Nossal NG. J Biol Chem. 1982;257:12426–34.
46. Matson SW, Tabor S, Richardson CC. The gene 4 protein of bacteriophage T7. Characterization of helicase activity. J Biol Chem. 1983;258:14017–24.
47. Dillingham MS, et al. Fluorescent single-stranded DNA binding protein as a probe for sensitive, real-time assays of helicase activity. Biophys J. 2008;95(7):3330–9.
48. Eggleston AK, Rahim NA, Kowalczykowski SC. A helicase assay based on the displacement of fluorescent, nucleic acid-binding ligands. Nucleic Acids Res. 1996;24:1179–86.
49. Ali JA, Lohman TM. Kinetic measurement of the step size of DNA unwinding by Escherichia coli UvrD helicase. Science. 1997;275(5298):377–80.
50. Yodh JG, Schlierf M, Ha T. Insight into helicase mechanism and function revealed through single-molecule approaches. Q Rev Biophys. 2010;43(2):185–217.
51. Comstock MJ, Ha T, Chemla YR. Ultrahigh-resolution optical trap with single-fluorophore sensitivity. Nat Methods. 2011;8(4):335–40.
52. Dessinges MN, et al. Stretching single stranded DNA, a model polyelectrolyte. Phys Rev Lett. 2002;89(24):248102.
53. Perkins TT, et al. Forward and reverse motion of single RecBCD molecules on DNA. Biophys J. 2004;86(3):1640–8.
54. Spies M, et al. A molecular throttle: the recombination hotspot chi controls DNA translocation by the RecBCD helicase. Cell. 2003;114(5):647–54.
55. Sun B, et al. ATP-induced helicase slippage reveals highly coordinated subunits. Nature. 2011;478(7367):132–5.
56. Fazio TA, et al. Fabrication of nanoscale “curtain rods” for DNA curtains using nanoimprint lithography. J Vac Sci Technol A. 2009;27(6):3095–8.
57. Fazio T, et al. DNA curtains and nanoscale curtain rods: high-throughput tools for single molecule imaging. Langmuir. 2008;24(18):10524–31.
58. Finkelstein IJ, Visnapuu ML, Greene EC. Single-molecule imaging reveals mechanisms of protein disruption by a DNA translocase. Nature. 2010;468(7326):983–7.