Chapter

A Study of p53 Action on DNA at the Single Molecule Level

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Abstract

The transcription factor p53 searches for and binds to target sequences within long genomic DNA, to regulate downstream gene expression. p53 possesses multiple disordered and DNA-binding domains, which are frequently observed in DNA-binding proteins. Owing to these properties, p53 is used as a model protein for target search studies. It counters cell stress by utilizing a facilitated diffusion mechanism that combines 3D diffusion in solution, 1D sliding along DNA, hopping/jumping along DNA, and intersegmental transfer between two DNAs. Single-molecule fluorescence microscopy has been used to characterize individual motions of p53 in detail. In addition, a biophysical study has revealed that p53 forms liquid-like droplets involving the functional switch. In this chapter, the target search and regulation of p53 are discussed in terms of dynamic properties.

Keywords: p53, single molecule, fluorescence, DNA, disordered, diffusion, jumping, intersegmental transfer, sliding, hopping, target search, liquid–liquid phase separation

1. Introduction

p53 is a multifunctional transcription factor that induces cell cycle arrest, DNA repair, and apoptosis, thereby suppressing cell cancerization [1, 2]. It is referred to as a guardian of the genome that determines cell fate. When p53 is activated by various stress factors, it searches for and binds to target DNA sequences and regulates the expression of downstream genes. p53 is composed of an N-terminal (NT) domain, core domain, linker, tetramerization (Tet) domain, and C-terminal (CT) domain. The core and Tet domains possess specifically folded structures, while other domains are intrinsically disordered [3–5]. p53 forms a tetramer via Tet domains [5]. Core and CT domains are involved in its binding to DNA sequences in a specific and nonspecific manner, respectively [6]. Fifty percent of gene mutations in tumor cells were found in p53, and many of the identified mutations were located in structured domains, which inhibited target DNA binding [3]. Comprehensive mutagenesis analysis supports the correlation between the structured domains of p53 and its function [7]. Since p53 possesses common properties frequently observed in DNA-binding proteins, including oligomerization, disordered regions, and multiple DNA-binding domains [8], it is used as a model protein in the target search study described below [9–11].

The target DNAs for p53 were ~ 20 bp, while the genomic DNA was ~10^9 bp. Accordingly, p53 was required to search for small targets efficiently from within vast lengths of non-target DNAs. This is known as a target search problem for

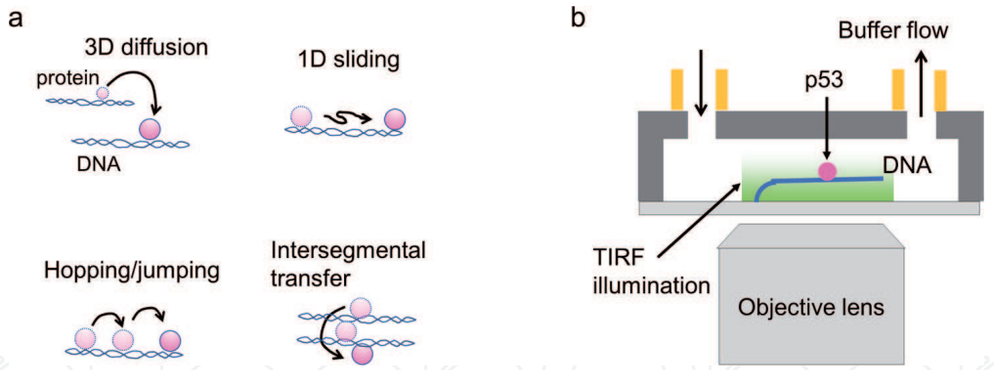

Figure 1.
Target search dynamics of DNA-binding proteins and visualization of p53 dynamics on DNA by single-molecule fluorescence microscopy. (a) Schematic diagram of four target search dynamics. (b) Schematic diagram of single-molecule fluorescence microscope and flow cell. In the flow cell, one end of the DNA is tethered to the surface and it is stretched by buffer flow. p53 molecule labeled to a fluorescence dye is illuminated by TIRF and the fluorescence is detected by EM-CCD through an objective lens. Panels (a) and (b) are adapted from ref. [12] and ref. [13] with some modifications, respectively.

sequence-specific DNA-binding proteins. To solve this problem, a facilitated diffusion mechanism has been proposed for DNA-binding proteins. The facilitated diffusion is the integration of three-dimensional (3D) diffusion in solution, one-dimensional (1D) diffusion along DNA, hopping/jumping along DNA, and intersegmental transfer between two DNAs (**Figure 1a**). In 3D diffusion, p53 diffuses in solution, altering the search sites on genomic DNA. In 1D sliding, it moves along the DNA, while maintaining continuous contact. In addition, p53 hops or jumps along DNA (within 100 bp of jump). Intersegmental transfer enables p53 to move from one DNA to another without dissociation. Theoretical studies suggest that the integration of multiple search dynamics, while not requiring all dynamics, can facilitate the target search [14–17]. The facilitation factor depends on various physical parameters, such as diffusion coefficient along DNA, residence time on DNA, dissociation time in solution, and frequency of transfer and jump.

How does p53 solve the target search problem using facilitated diffusion? How is the target search and binding of p53 regulated? In this chapter, I explain the facilitated diffusion and regulation of p53 based on recently accumulated single-molecule data.

2. Single-molecule fluorescence microscopy

Single-molecule fluorescence microscopy enables the differentiation and characterization of individual search dynamics of DNA-binding proteins, including p53, as reported previously [18–24]. In general, the system combines a fluorescence microscope and a flow cell (**Figure 1b**). In the flow cell, one end of the DNA is tethered to the surface, and it is stretched by buffer flow. Several methods have been proposed for tethering DNAs [18, 25–29]. For example, a DNA garden is a simple method for producing DNA arrays, in which neutravidin molecules are printed in a line on polymer-coated coverslips, and biotinylated DNAs are tethered to the printed neutravidin [29]. p53 molecules labeled with a fluorescence dye are introduced into the flow cell using a syringe pump. The fluorescent p53 bound to DNA is selectively illuminated by total internal reflection fluorescence (TIRF). p53 molecules on DNA are detected as fluorescent spots on the sequential images of an electron-multiplying charge-coupled device (EM-CCD). The positions of molecules were tracked using an appropriate analysis program to visualize the search dynamics of p53.

3. Target search dynamics of p53

In 2008, 1D sliding of p53 along DNA was observed for the first time using single-molecule fluorescence microscopy [30]. This observation was consistent with a reported indirect evidence that p53 dissociated rapidly from short DNA in the absence of blocks at ends by sliding off from DNA [31]. In 2011, a study of p53 mutants deleting either of two DNA-binding proteins revealed that p53 can slide along DNA using disordered CT domains [32]. This is consistent with the fact that a designed peptide targeting CT domains suppressed the 1D sliding of p53 [33]. Furthermore, 1D sliding of p53 was supported by molecular dynamics simulations [34, 35]. In 2012, it was shown that 1D sliding dynamics of p53 depends slightly on DNA sequence, suggesting that p53 feels the energy landscape based on DNA sequence through interactions between core domains and DNA [36]. In 2015, a detailed analysis of 1D sliding dynamics demonstrated that p53 possesses two sliding modes on non-target DNA [37, 38]. In the fast mode, it interacts with DNA loosely using CT domains. In contrast, in the slow mode, it binds tightly to DNA using core and CT domains (**Figure 2a**). In 2017, the disordered linker was

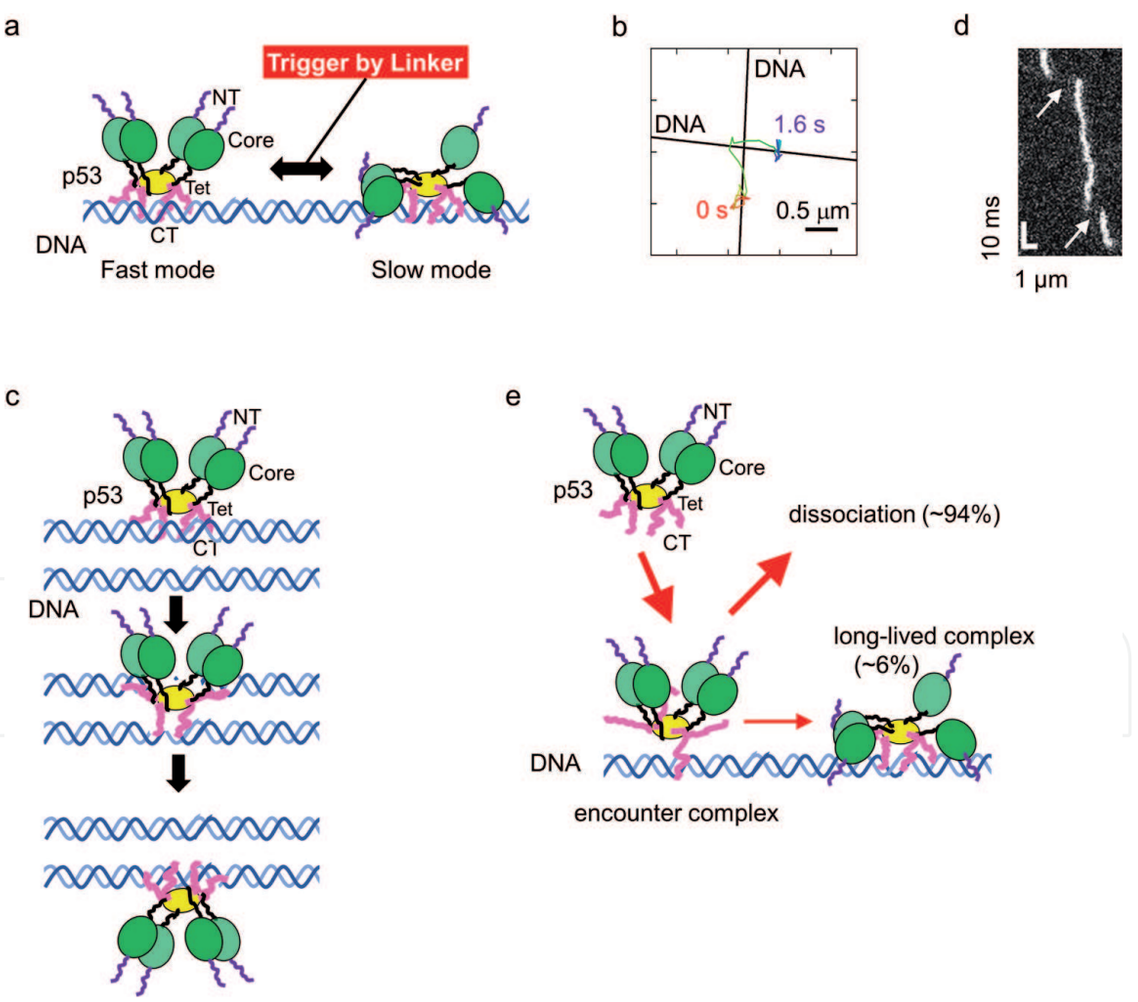

Figure 2.
Target search dynamics of p53. (a) Schematic diagram of two modes for 1D sliding p53 along DNA. p53 is composed of the NT (purple), Core (green), linker (black), Tet (yellow), and CT (pink) domains. The switch between two modes is triggered by the linker. (b) Typical single-molecule data showing intersegmental transfer of p53 between crisscrossing DNAs. (c) Schematic diagram of intersegmental transfer of p53 between two DNAs. p53 uses CT domains (pink) for the transfer. (d) Typical single-molecule data showing jumping of p53 along DNA (white traces). Arrows denote the jumping events. (e) Schematic diagram of encounter complex formation of p53 and conversion from the encounter complex to long-lived complex. Panel (b) is adapted from ref. [39] with some modifications. Panels (d) and (e) are adapted from ref. [40] with some modifications.

identified to trigger the switch between the two modes (**Figure 2a**) [41]. In 2016, the target recognition process of p53 was characterized in detail [42]. The results demonstrated that target recognition occurs mainly via 1D sliding. The target recognition of p53 was quite low (the successful recognition probability was 7%), but it was enhanced two-fold upon a post-translational modification. Accordingly, 1D sliding is considered as one of the important dynamics in the target search and binding of p53.

In 2018, intersegmental transfer of p53 was examined using ensemble kinetic and single-molecule fluorescence measurements [39]. After the solutions of p53 bound to fluorescently labeled DNA and non-labeled DNA were mixed, the transfer reaction of p53 was monitored between the two DNAs. The observed reactions included the dissociation of p53 from one DNA and its transfer to the other. Actually, as the concentration of non-labeled DNA increased, the observed rate constant increased, suggesting intersegmental transfer. The rate constant of the transfer was ~10^8 M^{-1} s^{-1}, which is close to the diffusion limit. Furthermore, single-molecule tracking of p53 on crisscrossed DNAs demonstrated that p53 moves along the first DNA and then moves along the second DNA through the transfer at the intersection (**Figure 2b**). A study of p53 mutants deleting either of two DNA-binding domains identified that p53 binds to the first DNA and then to the second DNA using disordered CT domains at the same time; it then releases the first DNA, resulting in a transfer between the two DNAs (**Figure 2c**). This mechanism is supported by molecular dynamics simulations of p53 [43].

In 2020, the hopping/jumping of p53 on DNA was investigated [40]. Hopping/jumping was expected to occur at a time scale that is faster than the time resolution of the microscope (ex. 33 ms). To detect these events, the time resolution of the microscope was improved to 500 μs by optimizing the fluorescence excitation based on critical angle TIRF illumination and by utilizing the time delay integration mode of the EM-CCD [40]. Using the sub-millisecond-resolved microscope, jumping events of p53 along DNA were directly detected (arrows in **Figure 2d**). The jump frequency of p53 was ~6 s^{-1}, and the jump time was 2.2 ms. Based on the study of p53 mutants deleting either of two DNA-binding domains, disordered CT domains were identified to be indispensable for the jumping of p53 along DNA [13]. Furthermore, 1D diffusion along DNA was enhanced upon increasing the salt concentration, suggesting that p53 moves along DNA by hopping DNA-binding domains. Thus, it was revealed that p53 possesses hopping and jumping dynamics along DNA.

In 2016, 3D diffusion of p53 was characterized using ensemble kinetic measurements [42]. Association rate constants for target and non-target DNAs were determined to be ~10^9 M^{-1} s^{-1}, comparable to the diffusion limits. The difference in affinity for target and non-target DNAs was attributed to the dissociation rate constants. In 2020, the association process of p53 with non-target DNA was further investigated at the single-molecule level using a sub-millisecond resolved fluorescence microscope [40]. Kymographs demonstrated that short-lived traces of p53 with an average residence time of 2.8 ms were detected in addition to long-lived traces moving along DNA. The short-lived complex was interpreted as an encounter complex. Disordered CT domains of p53 were identified to participate in the transient complex formation and in the conversion from the transient complex to the long-lived complex [13] (**Figure 2e**). The long-lived complex was further stabilized by core domains [13].

Overall, single-molecule fluorescence microscopy revealed that p53 possesses all four search dynamics proposed theoretically: 3D diffusion, 1D sliding, hopping/jumping, and intersegmental transfer. The unique structure of p53, which is a tetramer of two DNA-binding domains, enables these search dynamics. This is the first study to examine all search possibilities for a single model protein.

4. Liquid–liquid phase separation of p53

Target search and binding of p53 might be regulated by a liquid-like assembly of p53 molecules. In a liquid–liquid phase separation (LLPS), p53 molecules, which disperse in the bulk phase, assemble and form a condensed phase called liquid droplets. In the droplet phase, p53 can move fluidly while maintaining a high concentration. This fluid property in the condensed phase differs from the solid aggregation that causes malfunction of p53. Early *in vivo* studies demonstrated that p53 is recruited into cellular droplets such as Cajal and promyelocytic leukemia protein (PML) bodies [44–46]. These facts suggest that LLPS might be involved in the cellular functions of p53.

In 2020, this possibility was extensively examined using *in vitro* measurements such as scattering, DIC microscopy, and fluorescence microscopy [47]. p53 formed micrometer-sized droplets at neutral and slightly acidic pH and low salt concentrations. The fusion events of at least two droplets into a single large droplet were observed, confirming the fluidity of p53 inside the droplets (**Figure 3a**). High fluorescence intensity was detected in the droplets of p53 labeled with a fluorescent dye, supporting the high concentration of p53 in the droplets (**Figure 3b**). The droplet formation of p53 was affected by pH and salt concentrations. This suggests that attractive electrostatic interactions among local parts of p53 and repulsive net charges among whole molecules of p53 are balanced, resulting in droplet formation. Deletion of either of the disordered NT and CT domains suppressed the droplet formation of p53. This suggests multivalent electrostatic interactions between the oppositely charged NT and CT domains in p53 droplets.

The structural properties of p53 in solution and in droplet form were investigated using fluorescence resonance energy transfer (FRET) between two fluorophores labeled at two residues of p53. Since FRET depends on the distance

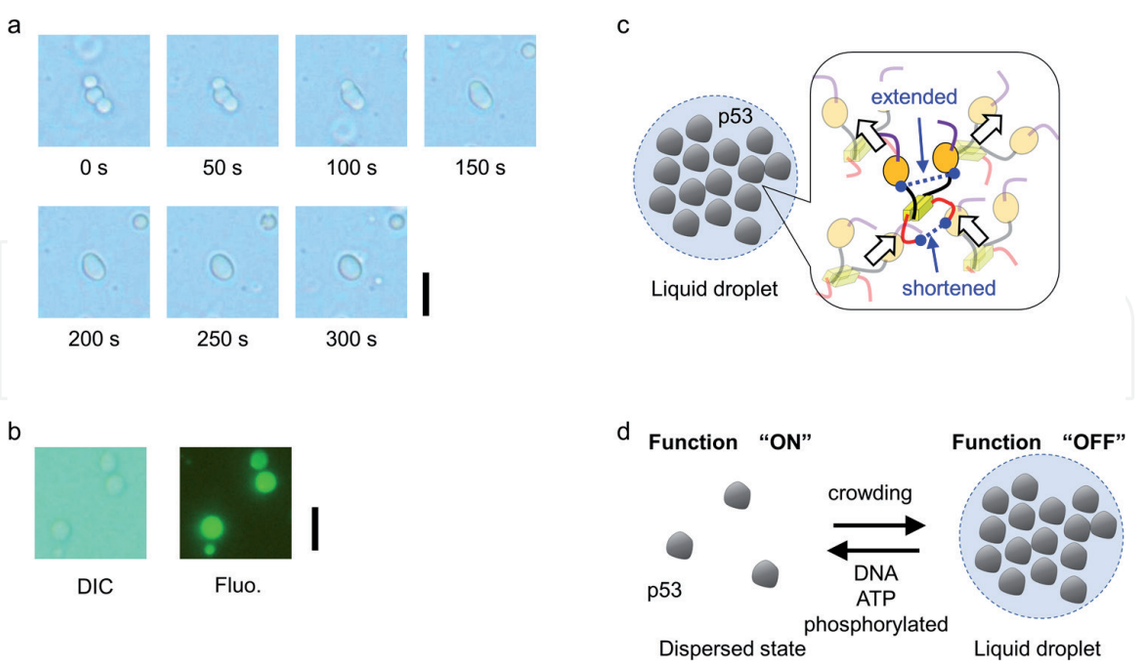

Figure 3.
Liquid droplet formation of p53 regulates its function. (a) Time course of a typical fusion event of three p53 droplets into a single droplet using DIC microscopy. (b) DIC and fluorescence images of the droplets of Alexa488-labeled p53 and non-labeled p53. Scale bars in panels (a) and (b) represent 10 μm. (c) Schematic diagram of p53 conformation in the droplet. p53 is composed of the NT (purple), Core (orange), Tet (yellow), and CT (red) domains. In the droplets, the NT and CT domains interact electrostatically. Arrows denote the structural changes on the different domains of p53 that are induced by the intermolecular interactions in a droplet. The dimer structure is displayed for clarity. (d) Functional switch model of p53. The panels (a)-(d) are adapted from ref. [47] with some modifications.

between the two fluorophores, it was used to measure the conformational changes. The distance between the core domains of p53 was slightly longer in the droplets, while the distance between the CT domains became slightly shorter (**Figure 3c**). Accordingly, p53 adopted a new tertiary structure, forming interactions with the adjacent molecules in the droplets.

Does p53 maintain binding to the target DNA after experiencing the droplet formation? The reactions of p53 binding to the target DNA were similar before and after the droplet formation. These results indicate that droplet formation of p53 is reversible, and p53 dispersed in solution from the droplets retains its DNA binding ability.

Droplet formation of p53 was found to be regulated by molecular crowding, endogenous molecules, and post-translational modification. Molecular crowding agents, mimicking the cellular crowding condition, promoted droplet formation. In contrast, ssDNA, dsDNA, and ATP suppressed it. The p53 mutant mimicking post-translational phosphorylation did not form droplets. Based on these results, a functional switch model was proposed (**Figure 3d**). Under normal cell conditions, the compartmentalization of p53 into the droplets suppresses its function as a transcriptional regulator. Under stress conditions, the activation of p53, triggered by posttranslational phosphorylation, releases p53 from the droplets and promotes target search and binding.

5. Target search and regulation model of p53

In this section, the current model of p53 is described in terms of target search and regulation. p53 functions as a transcription factor that responds to various emergency situations in cells. Under normal cell conditions, p53 turns off through the following mechanisms. First, the copy number of p53 is maintained at a low level, allowing dimers with a low affinity to target DNAs in an oligomeric state [48, 49]. Second, post-translational modifications for activating p53 are not added, for example, suppressing the target recognition of p53 [42]. Third, p53 is stored in liquid droplets [47]. These actions of p53 prevent its malfunction under normal conditions.

Under cellular stress, p53 is activated by post-translational modifications [1, 2, 50–56] and by a change in its oligomeric state from dimers to tetramers, with a high affinity for target DNAs [48, 57–59]. Phosphorylation of the CT domain of p53 triggers its release from the droplets, allowing it to engage in target search [47]. The increase in the copy number of p53 also facilitates the target search [60]. As explained above, p53 utilizes facilitated diffusion combining four search dynamics. Using 3D diffusion, p53 associates randomly with nonspecific sites of DNA, followed by dissociation. Until p53 associates with the target sequence by chance, it repeats such association and dissociation motions. If the search motion of p53 is limited to 3D diffusion, it would be a time-consuming endeavor. After p53 associates with the nonspecific site of DNA by 3D diffusion, it can search for the target sequence along DNA near the bound site using 1D sliding and hopping/jumping. The search distance of p53 per association event is estimated to be 700 bp [40], corresponding to approximately 35-fold facilitation of the target search.

In cells, genomic DNAs are covered by many DNA-binding proteins, including histones and other nucleoid proteins. These DNA-binding proteins may act as obstacles in the target search of p53. For example, when the sliding p53 collides with other DNA-binding proteins on DNA, it may not be able to bypass these obstacles due to steric hindrance, thereby limiting the search distance on DNA. However, p53 possesses two bypass mechanisms: the jumping along DNA [40] and the intersegmental transfer between two DNAs [39]. Using these motions, it can overcome such obstacles and continue its search for targets in cells. Overall, the search and regulation strategies of p53 could satisfy various cellular requirements.

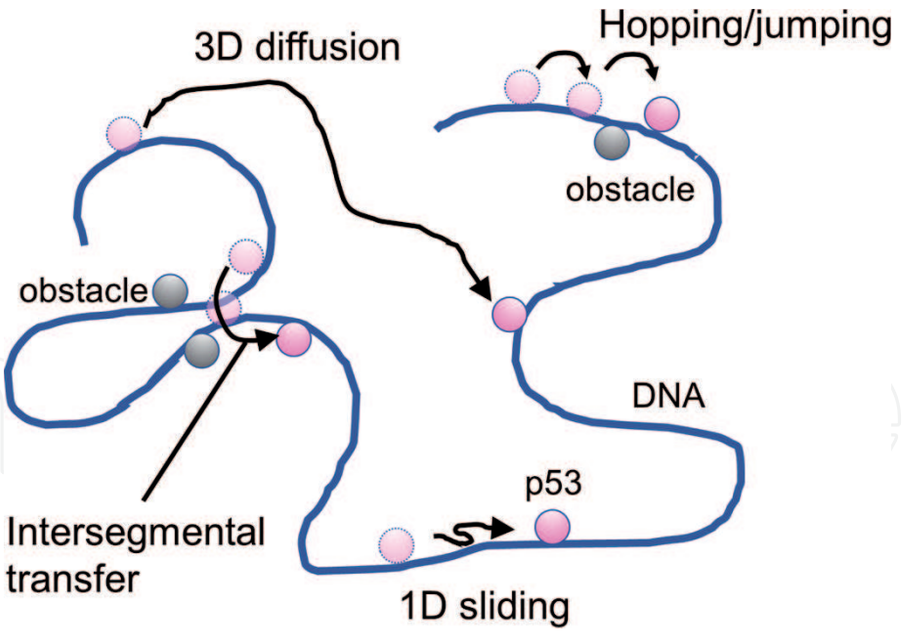

Figure 4.
Schematic diagram of target search of p53. Pink and gray circles are p53 and obstacle bound to DNA, respectively. Four search mechanisms are illustrated.

6. Conclusions

The target search and binding of p53 and its regulation have been characterized using single-molecule fluorescence microscopy and relevant biophysical methods. The accumulated data demonstrate that p53 searches for target DNAs utilizing four search dynamics: 3D diffusion in solution, 1D sliding along DNA, hopping/jumping along DNA, and intersegmental transfer between two DNAs (**Figure 4**). Especially, hopping/jumping and intersegmental transfer between two DNAs are required to bypass obstacles bound to DNA. It was reported that other DNA-binding protein with a disordered DNA-binding domain bypasses obstacles through obstacle-unbound region of DNA [24]. Since p53 possesses a similar disordered DNA-binding domain, it is not surprising that p53 possesses this bypass mechanism. Target search and binding are regulated by copy number, post-translational modifications, and liquid droplet formation. Considering that p53 can interact with many partner proteins, the partner proteins may affect the target search. Complexity in the target search and regulation of p53 would enable a response to various emergency situations in cells and be required to satisfy various cellular requirements.

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