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5 **One-dimensional sliding of p53 along DNA is accelerated in the presence of Ca^{2+} or Mg^{2+} at**
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8 **millimolar concentrations**
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Abstract: One-dimensional (1D) sliding of the tumor suppressor p53 along DNA is an essential dynamics required for its efficient search for the binding sites in the genome. To address how the search process of p53 is affected by the changes in the concentration of Mg^{2+} and Ca^{2+} after the cell damages, we investigated its sliding dynamics at different concentrations of the divalent cations. The 1D sliding trajectories of p53 along the stretched DNA were measured by using single-molecule fluorescence microscopy. The averaged diffusion coefficient calculated from the mean square displacement of p53 on DNA increased significantly at the higher concentration of Mg^{2+} or Ca^{2+} , indicating that the divalent cations accelerate the sliding likely by weakening the DNA-p53 interaction. In addition, two distributions were identified in the displacement of the observed trajectories of p53, demonstrating the presence of the fast and slow sliding modes having large and small diffusion coefficients, respectively. A coreless mutant of p53, in which the core domain was deleted, showed only a single mode whose diffusion coefficient is about twice of that of the fast mode for the full-length p53. Thus, the two modes are likely the result of the tight and loose interactions between the core domain of p53 and DNA. These results demonstrated clearly that the 1D-sliding dynamics of p53 is strongly dependent on the concentration of Mg^{2+} and Ca^{2+} , which maintains the search distance of p53 along DNA in cells that lost homeostatic control of the divalent cations.

Keywords: p53; single-molecule; DNA; sliding; divalent cation

Abbreviations: 1D, one-dimensional; TIRF, total internal reflection fluorescence; 3D, three-dimensional; spDNA, the specific target sequence of dsDNA; nspDNA, the non-specific sequence of dsDNA; ST7, thermostable mutant

Introduction

The tumor suppressor p53 is the transcription factor that controls the cellular fate as a guardian of the genome [1,2]. p53 induces cell cycle arrest, apoptosis, or DNA repair upon different types of cellular stresses. p53 has four domains; N-terminal domain, core domain, tetramerization domain, and C-terminal domain. A flexible linker connects the core domain and the tetramerization domain. The core and tetramerization domains have specific folded structures, while the other domains are disordered. The core and C-terminal domains are respectively related to the binding of p53 to DNA in sequence-specific and nonspecific manners. The specific binding of p53 to the target DNA sequence causes the transactivation of gene products leading to the various cell fates. In 50% of human cancers, the mutations of p53 gene are found, which are located in the core domain and prevent the specific binding to DNA [3]. Due to its importance, the mechanism by which p53 searches for its binding sites among huge DNA has been the focus of intensive investigations [4-8].

The one-dimensional (1D) sliding of p53 along DNA is one of the essential dynamics in the search process and has been directly investigated by single-molecule techniques. The sliding of p53 along the fixed DNA was first observed using a high-speed atomic force microscopy [4]. Subsequently, the sliding of p53 was observed using fluorescence microscopy by tracking the position of fluorescent-labeled p53 bound to DNA stretched by a flow [5,6]. The diffusion coefficient for the sliding dynamics of p53 was determined and was $0.16 \mu\text{m}^2/\text{s}$ [5]. The C-terminal domain promotes the diffusion along the DNA, while the core domain suppresses the diffusion [5]. The diffusion coefficient

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5 does not depend on the concentration of a monovalent cation, K^+ [5,6]. The DNA sequence slightly
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8 affects the diffusion [7]. It is proposed that p53 diffuses along DNA and searches for the target sites
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11 by repeating the transient association of the core domains to DNA and their dissociation, which was
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14 supported by the course-grained MD simulations [8]. These results demonstrate the importance of the
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17 sliding dynamics of p53 along DNA.
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20 The sliding dynamics of p53 might be regulated by the cellular conditions in various cell
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23 phases. For example, the cell death is deeply linked to Ca^{2+} signaling [9,10]. Under some stress, the
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26 expression level of p53 is elevated and the Ca^{2+} concentration is increased [11,12]. The elevated
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29 concentration of Ca^{2+} in nucleus induces chromatin unfolding, promotes the gene transcriptions
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32 regulated by Ca^{2+} binding proteins, and the digestion of nuclear DNA by Ca^{2+} and Mg^{2+} -dependent
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35 endonucleases. In addition, Mg^{2+} deficiency induces the elevated expression of p53, resulting in cell
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38 cycle arrest [13]. Since these divalent cations bind tightly and specifically to DNA [14-19], the sliding
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41 dynamics of p53, dictated by the interaction between p53 and DNA, would also be modulated by the
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44 divalent cations. In fact, early investigations of Lac repressor suggested that Mg^{2+} alters the 1D sliding
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47 along DNA and the binding ability to DNA [20,21]. Accordingly, we hypothesize that the loss of
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50 homeostatic control for divalent cations such as Ca^{2+} and Mg^{2+} might regulate the target search of p53.
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53 In this study, we investigated the effect of the divalent cations on the sliding dynamics of
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56 p53 along DNA. To this end, we prepared a stable tetrameric mutant of p53 (the pseudo WT) that can
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59 maintain the specific binding ability to DNA sufficient for the single-molecule fluorescence
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5 measurements. Using the pseudo WT, the nonspecific binding ability relevant to the sliding dynamics
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8 was examined. We found that the dissociation rate constant was increased as the increase in the
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10 concentration of Mg^{2+} or Ca^{2+} . We observed the sliding of p53 along DNA at the single molecule level
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13 at different concentrations of Mg^{2+} and Ca^{2+} , and found that the sliding of p53 was significantly
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16 accelerated by adding the millimolar concentration of the divalent cations. The analysis of the sliding
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19 trajectories further suggests that p53 possesses two sliding modes with different diffusion coefficients.
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22 We also prepared a coreless mutant of p53, in which the core domain and the N-terminal domain were
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25 deleted, and found that a single diffusion mode was observed in the coreless mutant. We propose that
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28 the increased diffusion of p53 in the presence of the divalent cations occurs as the result of a
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31 weakening of the DNA-p53 interaction upon the binding of the divalent cations to DNA.
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Results

Construction of the stabilized mutant of p53 with a single exposed cysteine

In our early trials, we prepared the human wild-type p53 by expressing the protein with GST-tag in *E. coli*, followed by the cleavage of the tag and the purification. The oligomeric state of the purified protein was examined by gel filtration, SDS-PAGE for the cross-linked p53 and native PAGE. In addition, the DNA binding activity was examined by electrophoretic mobility shift assay. We succeeded in the preparation of the tetrameric p53 having the specific binding ability towards the target DNA; however, it was difficult to reproduce the activity especially after frozen storages. This is likely due to the aggregation-prone property and/or the incomplete folding of the core domain of the wild-type p53 [22]. In addition, the labeling of the wild-type p53 by fluorophore for the single-molecule measurements likely affected its activity, because some of the four solvent-exposed cysteines are located in the binding site for DNA (Supplementary fig. 1 and Supplementary text). We concluded that the wild-type p53 is not suitable for the investigation based on the single-molecule fluorescence measurements. The instability of the wild-type p53 was similarly reported in previous investigations [5,22,23].

To obtain reproducible results, we prepared a thermostable mutant having a single solvent-exposed cysteine for the labelling. The mutations introduced are C135V, C141V, W146Y, C182S, V203A, R209P, C229Y, H233Y, Y234F, N235K, Y236F, T253V and N268D, which are based on thermostable mutations reported as ST7 [23]. C176, C238 and C242 are left intact due to their

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5 involvement in coordination to Zn^{2+} , which is required for the binding of p53 to DNA. C124A,
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8 C275A and C277A are introduced to remove the rest of the cysteines, and K292C is introduced as the
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10 single labeling site. We name the thermostable mutant pseudo wild type (WT). The pseudo-WT p53
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13 with GST-tag expressed in *E. coli* was purified after cleavage of the tag. The yield of the purified
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16 mutant was 1.7 mg per 1 L of culture, which was improved significantly compared with the yield, 0.4
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19 mg per 1 L of culture, of the wild-type p53. The increased yield is likely the result of the codon
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22 optimization for *E. coli* and the increased stability of the mutant. Based on the SDS-PAGE analysis
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25 for the sample cross-linked by glutaraldehyde, we confirmed that the pseudo WT formed a tetramer
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28 (Supplementary fig. 2a). In addition, the molecular weight of the pseudo WT estimated by the native
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31 PAGE assay was consistent with that of the tetramer obtained in gel filtration [24] (Supplementary fig.
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34 2b). The pseudo-WT p53 was labeled with a fluorescence dye, ATTO532, at the labeling efficiency of
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37 1.4 dyes per monomer.
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44 examinations. First, we performed electrophoretic mobility shift assay (Supplementary fig. 2c and
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47 Supplementary text). The purified p53 was strongly bound to the specific target sequence with 30 bp
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50 (spDNA). In contrast, in the presence of a competitor that is 100 bp of dsDNA without the target site,
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53 the amount of the p53-spDNA complex was suppressed. Furthermore, in the presence of an antibody
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56 PAb421, which binds to the C-terminal domain of p53 and enhances the binding of p53 to spDNA, the
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59 p53-spDNA-PAb421 complex was observed even in the presence of the competitor. These results are
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consistent with those of a previous report [25]. Second, we performed the titration of the fluorescence anisotropy of FAM attached to spDNA and 30 bp of a non-specific sequence of dsDNA (nspDNA) at different concentrations of the p53 mutant [23,26]. The anisotropies of spDNA and nspDNA labeled by FAM showed the saturation behavior at the increased concentration of the p53 mutant, suggesting the 1:1 binding (Fig. 1a). The anisotropy data were well fitted by a two-state model, in which one tetramer of p53 binds to one DNA with an apparent dissociation constant (see Eqs. (3) and (4) in Materials and Methods). To prevent the dissociation of the tetramer, all the measurements in this study were performed within 50 min after the dilution of the stock solution [27]. The apparent dissociation constants for the binding of p53 to spDNA and nspDNA were 0.8 ± 0.1 and 8 ± 1 nM per tetramer, respectively. Taking the difference in the experimental conditions such as salt concentration into account, we conclude that the apparent dissociation constant for spDNA is consistent with the reported values, which were on the order of nM [23,26]. The observation that the apparent dissociation constant for spDNA is lower compared to that for nspDNA indicates that the pseudo-WT p53 retained the ability to bind the target DNA sequence specifically.

We confirmed that the oligomeric state and the activity of the pseudo-WT p53 were not altered even after the frozen storages (not shown). Thus, these results demonstrated that the constructed p53 is stable and possesses the specific DNA binding ability, and is suitable for the further investigations based on fluorescence microscopy.

Effect of the divalent cations on the nonspecific binding of p53 to DNA

To understand possible roles of the divalent cations on the 1D sliding dynamics of p53 along non-specific DNA, we first examined the effect of the divalent cations on the equilibrium dissociation constant of p53 from nspDNA at the ensemble level. We performed the titration of the fluorescence anisotropy of FAM attached to nspDNA at different concentrations of the pseudo-WT p53 under various Mg^{2+} concentrations (Fig. 1b). The apparent dissociation constant obtained by fitting the anisotropy data was constant at the Mg^{2+} concentration below 1.5 mM, but increased above 2 mM (Fig. 1c), confirming that Mg^{2+} at the millimolar concentration affects the p53-DNA interaction. We also examined the effect of Ca^{2+} on the apparent dissociation constant, which was slightly decreased as the increase in $[Ca^{2+}]$, suggesting the effect of Ca^{2+} is different from that of Mg^{2+} (Fig. 1c and Supplementary fig. 3).

To further examine the effect of the divalent cations on the p53-DNA interaction, we constructed a stopped-flow fluorescence-anisotropy apparatus and measured the dissociation kinetics of the pseudo-WT p53 from nspDNA. The fluorescence anisotropy of the labeled nspDNA bound to p53 decreased after a mixing with an excess amount of spDNA with no fluorescent dye, corresponding to the dissociation of p53 from nspDNA (Fig. 2a). We fitted the obtained time courses with a single exponential, and obtained the apparent rate constant for the dissociation of p53 from nspDNA, k_{off} . The obtained k_{off} value in the absence of Mg^{2+} is consistent with the reported residence time, less than 1 s, of p53 on DNA [23]. As shown in Fig. 2b, the k_{off} value increased significantly

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5 with the concentration of Mg^{2+} . We further examined the effect of Ca^{2+} on the dissociation kinetics
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8 and observed that the k_{off} value increased similarly with the concentration of Ca^{2+} (Fig. 2b and
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11 supplementary fig. 4). Thus, these results clearly demonstrated that the divalent cations can accelerate
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14 the dissociation kinetics of p53 from DNA without the target sequence.

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17 We found that the divalent cations affect the nonspecific binding of p53. We propose that
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20 Mg^{2+} and Ca^{2+} may weaken the direct interaction between p53 and DNA, resulting in the acceleration
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23 in the dissociation of p53 from DNA. Considering the dependence of k_{off} on the concentration of
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26 divalent cations, we expect that the 1D sliding of p53 along DNA might be altered upon the increase
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29 in the concentration of the divalent cations.

30 31 32 33 34 35 **Single-molecule observation of the 1D sliding of p53 and the determination of the diffusion** 36 37 38 **coefficient** 39 40

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42 To observe the sliding of p53, the stretched DNA in the flow cell under flow pressure was
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45 prepared as follows. The flow cell consists of a coverslip, a glass slide, and a spacer [28]. The width
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48 and height of the flow path in the flow cell were 3-4 mm and 0.1 mm, respectively. The inner surface
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51 of the coverslip was coated by polyethylene glycol conjugated with biotin (Fig. 3a). The DNA with
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54 both biotinylated ends [29,30] was then introduced to the cell in the presence of neutravidin, and was
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57 attached on the coverslip at one end. λ DNA, which does not contain the target sites for p53, was used
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60 to investigate the sliding along non-specific DNA. The flow was applied to stretch the DNA at the
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5 volume flow rate of 500 $\mu\text{L}/\text{min}$. The averaged line speed of the solution in the flow cell was
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8 estimated to be ~ 24 mm/s. The flow rate at the DNA estimated from the mean distance (0.2 μm)
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11 between the DNA and the coverslip surface was ~ 280 $\mu\text{m}/\text{s}$ [6]. The mean extension of the DNA was
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14 estimated to be 80% from Weissenberg number (290) that represents the dimensionless flow strength
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17 [31]. We observed that 5-20 DNAs were lined up along the flow direction in the field of view with a
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20 diameter of 30 μm in total internal reflection fluorescence (TIRF) microscopy.
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23 The TIRF microscopy was constructed to visualize the 1D sliding of p53 along the stretched
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26 DNA. The excitation light from 532-nm laser was reflected by a dichroic mirror, and was introduced
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29 to a peripheral portion of an objective corresponding to *N.A.* at around 1.40. The optical configuration
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32 enabled the sample illumination with the total-internal reflection mode. The fluorescence was
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35 collected by the same objective, and was detected by TDI-EM CCD camera after passing through the
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38 dichroic mirror. The labeled p53 was introduced into the flow cell in the presence of 2-mM Mg^{2+} , and
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41 was imaged as fluorescent spots. Two-dimensional images were taken at 33-ms interval.
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44 To examine the spatial resolution of the current system, we first selected the spots adsorbed
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47 on the cell surface and analyzed their behaviors. Fig. 3b shows typical trajectories of the pseudo-WT
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50 p53 obtained by tracking the center of the fluorescent spots in the two-dimensional images observed
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53 at each time. While some of the spots showed the movement along the longitudinal (red) and
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56 transverse (blue) directions, the rest of the spots did not move (dotted), suggesting that the latter spots
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59 correspond to the adsorbed p53 on the surface. The spots that did not move in the longitudinal
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direction did not move in the transverse direction either (not shown), further confirming that the spots correspond to p53 directly attached to the cell surface. By tracking the center of the adsorbed spots and calculating the standard deviation of the center, the accuracy of the localization of the molecule in the current experiments was estimated to be $0.02\ \mu\text{m}$ on average ($N\sim 103$).

We performed the following data analysis to select p53 attached on DNA and sliding along it and to exclude p53 adsorbed on the cell surface. First, we obtained time series of x and y coordinates of all the fluorescence spots in the images, which respectively correspond to the longitudinal and traverse axes of DNA, and selected data having more than 15 data points. Second, we segmented the time series of y -coordinate into every 10 data points, and calculated the mean square displacements (MSD) at 165-ms interval for each segment. We term the values as local MSD. Third, we selected the trajectories in which all the values of the local MSDs were larger than $0.0015\ \mu\text{m}^2$. This criterion could remove the molecules adsorbed on the coverslip. We obtained 182 trajectories whose averaged time duration was 0.8 s in the presence of 2-mM Mg^{2+} . The red and blue traces shown in Fig. 2b correspond to the trajectories along the longitudinal and transverse directions, respectively, selected by the above criteria. These trajectories show much larger movements than those of the molecules adsorbed on the coverslip, further confirming the removal of the adsorbed molecules in the selected molecules. It is apparent that the longitudinal movement, which corresponds to the sliding along DNA, is much larger than the transverse movement.

The trajectories of p53 thus selected should reflect the fluctuation due to the sliding of p53 along DNA and the fluctuation of the singly-fixed DNA in the flow. To examine the sliding property of p53, we plotted the time dependence of MSD averaged over all the selected trajectories (Fig. 3c). The averaged MSD in the longitudinal axis shows a linear relationship against the time interval, confirming that the sliding of p53 is Brownian in nature. The averaged diffusion coefficient D , obtained by fitting the slope of the MSD plots with $2D$, was $0.181 \pm 0.006 \mu\text{m}^2/\text{s}$ and is consistent with the value in the previous report ($0.16 \mu\text{m}^2/\text{s}$) [5]. As we will explain below, a portion of the molecules was affected by the flow and showed a drift; however, the drift was not significant and the MSD showed mostly linear dependency on the short time intervals. In contrast, the transverse movement was highly restricted, which can be attributed to the fluctuation of the DNA [5]. The averaged MSD in the transverse axis was saturated within ~ 0.2 s, which corresponds to the relaxation time of the fluctuation of the DNA. In large part of the stretched DNA except for the free edge, the fluctuation in the longitudinal axis was reported to be less than or comparable to that in the transverse axis [5]. Thus, we conclude that the fluctuation of the DNA should not significantly contribute to the observed fluctuation of p53 along the longitudinal axis.

Overall, we established a method to characterize the sliding dynamics of p53 by using TIRF microscopy. In addition, the detailed analysis of the observed data characterized that the sliding of p53 occurred as a diffusive motion.

The increased concentration of divalent cations accelerates the 1D sliding of p53

To examine the effect of the divalent cations in the 1D sliding of p53, we performed the single-molecule measurements at various Mg^{2+} concentrations. Fig. 3b and Supplementary fig. 5 show typical trajectories of the pseudo-WT p53 attached on the DNA in the presence of 0~3-mM Mg^{2+} . The sliding in the longitudinal axis was enhanced, as the concentration of Mg^{2+} was increased to more than 2 mM. The averaged MSDs show a linear relationship against time, suggesting that the simple diffusion governs the sliding of p53 at different concentrations of Mg^{2+} (Fig. 4a). As shown in Fig. 4c, the averaged diffusion coefficient increased as the increase in the concentration of Mg^{2+} . The averaged diffusion coefficient in the presence of 2-mM Mg^{2+} was five times larger than that in the absence of Mg^{2+} . Thus, these results demonstrate that Mg^{2+} dramatically alters the sliding property of p53.

We next investigated whether the other divalent ion, Ca^{2+} , affects the 1D sliding of the pseudo-WT p53 or not. Fig. 4b and c show the Ca^{2+} dependence of the averaged MSD curve and the averaged diffusion coefficient, respectively. Similar to the observations for Mg^{2+} , the averaged diffusion coefficient increased in the presence of 0.7~1.2-mM Ca^{2+} . The increase of the diffusion coefficient occurred at the lower concentration of Ca^{2+} compared to that of Mg^{2+} . Thus, the changes in the concentrations of Ca^{2+} also alter the 1D sliding dynamics of p53 along DNA.

The loss of the homeostatic controls of the divalent cations in cells was observed in the early stage of apoptosis and cell cycle arrest [11-13]. In such situations, p53 searches for and binds to the target site resulting in apoptosis or cell cycle arrest. In this study, we found that the changes in the

concentrations of the divalent cations in the range experienced in the cells that lost homeostatic controls drastically alter the 1D sliding dynamics of p53. In contrast, the monovalent cation does not affect the diffusional motion in the sliding of p53 [5]. These results suggest that the changes in the concentrations of divalent cations might also regulate the search process of p53 for the binding site.

Heterogeneity of the sliding of p53

To further examine the mechanism by which the 1D sliding is controlled by divalent cations, we next plotted the distributions of displacements for the observed trajectories at the time interval of 165 ms and at different concentrations of Mg^{2+} (Fig. 5). If p53 shows a simple diffusion, a single Gaussian distribution should be observed in the histogram. If p53 shows drifts caused by the flow pressure, the shift of the peak center in the displacement distribution should be observed. Indeed, the observed distributions clearly deviated from the single Gaussian, and are likely the sum of two Gaussians. One of the possible reasons for this may be the heterogeneity for the sliding of p53. The deviation from the single Gaussian becomes more apparent at the longer time interval (Supplementary fig. 6). We could fit all the distributions by the sum of two Gaussians, each having different diffusion coefficients and drift velocities as expressed in Eq. (1):

$$P(\delta x) = \sum_{i=1}^2 \frac{A_i}{\sqrt{4\pi D_i \delta t}} \exp\left(-\frac{(\delta x + v_i \delta t)^2}{4D_i \delta t}\right) \quad (1),$$

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5 where δx , A_i , v_i and D_i are respectively the displacement in the time interval, the amplitude, the drift
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8 velocity and the apparent diffusion coefficient of the i_{th} component. The result of the fitting was
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10 shown in Fig. 5, and clearly demonstrates that the sliding dynamics of the pseudo-WT p53 is
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12 heterogeneous, and can be described by two components. We refer to these two components as the
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14 fast and slow modes depending on the diffusion coefficients.
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20 Fig. 6 shows the Mg^{2+} -concentration dependence of the apparent diffusion coefficient and
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22 the drift velocity for the fast and slow modes estimated from the fitting of the data in Fig. 5. The
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24 apparent diffusion coefficients estimated for the slow mode did not significantly depend on the Mg^{2+}
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26 concentration. In fact, the distribution width for the slow mode did not apparently depend on the time
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28 interval (Supplementary fig. 6), suggesting that the observed distribution for the slow mode might be
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30 ascribed to the fluctuation of the DNA and that p53 in the slow mode might be stacked on the DNA.
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32 The fluctuation along the traverse direction in the slow mode was larger than that of the adsorbed
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34 molecules, suggesting that a transient interaction of p53 to the cell surface is not the origin of the slow
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36 mode. In accordance with the interpretation, the drift velocity of the slow mode was almost 0. In
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38 contrast, as the concentration of Mg^{2+} increased, the apparent diffusion coefficient and the drift
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40 velocity of the fast mode were accelerated significantly. The fractions of the fast and slow modes were
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42 respectively $49 \pm 3\%$ and $51 \pm 3\%$ at 2-mM Mg^{2+} , and did not significantly depend on the concentration
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44 of Mg^{2+} (Supplementary fig. 7). We could not detect clear transitions between the fast and slow modes
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46 in the trajectories within 330 ms, suggesting that the transition will occur at the longer time interval.
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Supplementary fig. 8 demonstrated that the two modes were also present when Ca^{2+} was added. The diffusion coefficients and the fractions of the two modes observed at 0.7-1.2-mM Ca^{2+} were similar to those at 2-3-mM Mg^{2+} . Thus, the detailed analysis of the displacement distribution suggests that the two modes are present in the sliding dynamics of p53 along DNA, and that the fast mode shows the accelerated diffusion in the presence of Mg^{2+} and Ca^{2+} .

We conducted various control experiments to rule out the possibility that the observed two modes are caused by some artifacts. The observed two modes are not the result of the heterogeneity in the sample identity, in the oligomeric state, in the affinity to DNA nor in the sample labeling (Fig. 1, Supplementary fig. 2, 4, 9, 10 and Supplementary text). We can also rule out the transient adsorption of p53 to the cell surface as the possible origin of the slow mode (Supplementary text). These control experiments strongly suggest that the presence of the two modes should be ascribed to the inherent heterogeneity in the interaction between p53 and DNA.

The core domain of p53 causes the heterogeneity of the sliding

The core domain, which is responsible for the specific binding of p53 to the target sequence of DNA, also suppresses the 1D sliding [5], suggesting that the core domain interacts with DNA in the sliding process. It can be further assumed that heterogeneous interactions between the core domain and DNA might cause the two sliding modes. To examine the roles of the core domain in the heterogeneous sliding dynamics of p53, we constructed a coreless variant of p53, in which the core

and N-terminal domains were deleted from the pseudo-WT p53, and labeled the variant with ATTO532 (Supplementary text). The sliding dynamics of the coreless mutant in the presence of 2-mM Mg^{2+} was examined in the flow cell coated by 2-methacryloyloxyethyl phosphorylcholine polymer (MPC polymer) (Supplementary text). We analyzed 94 single-molecule trajectories of the coreless mutant and plotted their distribution of displacement at the time interval of 165 ms (Fig. 7a). In contrast to the heterogeneous distribution observed for the pseudo WT, the distribution for the coreless mutant becomes homogeneous and can be well fitted by a single Gaussian expressed in eq. (2):

$$P(\delta x) = \frac{A}{\sqrt{4\pi D\delta t}} \exp\left(-\frac{(\delta x + v\delta t)^2}{4D\delta t}\right) \quad (2).$$

The diffusion coefficient, D , estimated by the fitting was $0.54 \pm 0.02 \mu m^2/s$, and the drift velocity, v , was $0.81 \pm 0.07 \mu m/s$. The estimated diffusion coefficient was close to that obtained in ref. 5 ($0.776 \pm 0.098 \mu m^2/s$). We repeated the same measurements of the pseudo-WT p53 in the identical experimental condition (Fig. 7b), and reproduced the two distributions. The diffusion coefficients of the fast and slow modes for the pseudo-WT p53 were 0.26 ± 0.02 and $0.03 \pm 0.02 \mu m^2/s$, respectively. The drift velocities of the fast and slow modes were respectively 0.51 ± 0.06 and $0.02 \pm 0.02 \mu m/s$. These values of the pseudo-WT p53 in the cell coated by MPC-polymer coating were identical to

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5 those in the cell coated by PEG coating (Fig. 6). These results demonstrate clearly that the core
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11 The diffusion coefficient of the coreless mutant, $0.54 \pm 0.02 \mu\text{m}^2/\text{s}$, which is ca. two times
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14 larger than that of the fast mode of the pseudo WT obtained in the identical condition (0.26 ± 0.02
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17 $\mu\text{m}^2/\text{s}$). It suggests that the core domain interacts weakly with DNA in the fast mode. In contrast, the
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20 diffusion coefficient of the coreless mutant was 19 times larger than that of the slow mode, suggesting
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23 that the core domain interacts strongly with DNA and retards the sliding dynamics of p53 in the slow
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26 mode. We propose that the loose and tight contacts of the core domains to DNA might be the origin of
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Discussion

In this investigation, we prepared the pseudo-WT p53 and examined the effect of the divalent cations on the 1D-sliding dynamics along the stretched DNA. The effect of monovalent cations on the diffusion coefficient was examined previously for several DNA binding proteins including p53 [5,32,33], and it was demonstrated that the potassium ion does not strongly affect the sliding dynamics of p53 [5]. In addition, early investigations demonstrated indirectly a possible role of Mg^{2+} in the sliding of the lac repressor [20,21]. However, there is no investigation so far to examine the effect of the divalent cations on the sliding process of p53. Since the concentration of the divalent cations changes significantly in cells that lost homeostatic control, the current investigation might provide new insight into the physiological function of p53. We will discuss the implication of the current observations below.

We will first compare the data obtained in the ensemble experiments and the data obtained by the single-molecule measurements. The ensemble stopped-flow experiments showed that the apparent dissociation rate constant of pseudo WT from 30bp nspDNA is $74 \pm 4 \text{ s}^{-1}$ in the presence of 2-mM Mg^{2+} (Fig. 2b). The analysis of the dwell time of pseudo WT on λ DNA obtained in the single-molecule measurements based on the flow cell coated by the MPC polymer showed a major component dissociating with the dissociation rate constant of $19 \pm 1 \text{ s}^{-1}$ and a minor component dissociating rather slowly (Supplementary fig. 11). The difference in the observations might be caused by the difference in the length of the DNAs used. It is also possible that the minor component

identified in the single-molecule measurements might be missed in the stopped-flow experiments. We next calculated the fraction of the fast and slow modes in the single-molecule trajectories with various dwell times by fitting the displacement distributions of the pseudo WT by Eq. (1). We confirmed that the fraction of the fast mode does not change (Supplementary fig. 12). Accordingly, we suggest that the fast and slow sliding modes of p53 possess the similar dissociation rate constants.

The target search process of p53 under different concentrations of the divalent cations

We examined the equilibrium dissociation constant and the kinetic dissociation rate constant of the pseudo-WT p53 from the non-specific sequence of DNA, and observed that the both equilibrium and kinetic properties of p53 depend on the concentration of the divalent cations. In particular, the apparent dissociation rate constant from the nspDNA increased for several times as we increase the concentration of Mg^{2+} and Ca^{2+} (Fig. 2b). In other words, the expected residence time of p53 on DNA should become shorter at the higher concentration of the divalent cations and might reduce the length of DNA that can be scanned by p53 in one binding event. We next clarified that the sliding dynamics of p53 becomes faster at the elevated concentration of the divalent cations (Fig. 4c). The faster sliding of p53 might accelerate the target search of p53. Thus, the divalent cations affect different steps of the target search process to the inverse directions.

To understand the overall effect of the divalent cations to the sliding dynamics of p53, we plotted the sliding distance of p53 on DNA, which is defined as the average size of DNA scanned per

one binding event (Supplementary text). Very impressively, the sliding distances of p53 estimated at various concentrations of the divalent cations are nearly constant and are within 140 bp to 250 bp, suggesting the shortened dissociation time at the elevated concentrations of the divalent cations is compensated for by the increased diffusion coefficient (Fig. 8). The sliding distances plotted should be considered as rough estimates and might underestimate the actual distances. To plot Fig. 8, we used the dissociation rate constants obtained in the ensemble experiment with the short DNA. As we discussed above, the ensemble rate constant obtained in the presence of 2-mM Mg^{2+} was several times faster than the rate constant estimated from the dwell time analysis of the single-molecule measurement in the corresponding condition (Supplementary fig. 11). The difference might be attributable to the dissociation of p53 from the ends of the short DNA in the ensemble experiment. However, the plot in Fig. 8 suggests that the target search of p53 per one binding event is maintained under different concentrations of the divalent cations.

The efficient search for the binding sites of DNA-binding proteins including p53 is considered to be achieved by a combination of 1D sliding and three-dimensional (3D) search [5,34,35]. The 3D search enables p53 to alter the search region of DNA by transiently dissociating from DNA and then reassociating to the different regions of DNA. The 1D sliding enables p53 to move along DNA without dissociation as the 1D diffusion. In addition, the accuracy of the recognition of the target DNA also controls the search process. Further investigations are necessary to understand the effect of the divalent cations to the different steps in the target search process of p53. However,

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5 this investigation clearly demonstrated the effect of the divalent cations for the 1D sliding process of
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10 11 12 13 14 **Mechanism of the accelerated diffusion of p53 in the presence of the divalent cations** 15

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17 Several possibilities can be proposed to explain the accelerated diffusion of p53 in the
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19 presence of the divalent cations. We note that there is no evidence supporting the specific binding of
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23 Mg^{2+} or Ca^{2+} to p53 expect for the binding of Mg^{2+} to the open binding site of Zn^{2+} [36]. In the
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26 experimental condition used in this study, however, Zn^{2+} should be tightly bound to p53, since an
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29 excess of EDTA at more than 5 mM is required to remove Zn^{2+} from p53 [37]. Thus, we assume that
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32 the divalent cations act to DNA primarily, and propose the following possible mechanisms in the
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39 First, the binding of the divalent cations to DNA might weaken the interaction between DNA
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41 and p53. Mg^{2+} and Ca^{2+} can bind specifically to phosphates and bases including guanine of DNA
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44 [14-19], and reduce the negative charges of the phosphate and may prevent the hydrogen bonds
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47 between the base and p53. Accordingly, the binding of the divalent cations to DNA might weaken the
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50 direct interaction between DNA and p53, resulting in the increase in the diffusion coefficient and the
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53 drift velocity of the fast mode, and the dissociation rate constant. It is also possible that the divalent
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p53 by the divalent cations might weaken the interaction and might accelerate the sliding [38]. However, the electrostatic screening of a monovalent cation does not affect the 1D sliding of p53 [5,6], suggesting the electrostatic screening does not likely modulate the interaction between p53 and DNA. Third, a conformational change of DNA caused by the binding of the divalent cations or by the binding of p53 might modulate the sliding process. It is known that the B form of DNA is maintained at different concentrations of Mg^{2+} or Ca^{2+} in the range from zero to several mM, except for a minor bending of the bases caused by cation- π interactions [39,40]. However, it is still possible that a small conformational change of DNA such as bending caused by the binding of the divalent cations or p53 alters the sliding dynamics of p53. Thus, the binding of the divalent cations to DNA and the subsequent weakening of the DNA-p53 interaction are likely the origin of the accelerated diffusion of p53.

We propose that the multiple sliding modes of p53 are caused by the different interactions in the complex of p53 and DNA. The C-terminal domain of p53 promotes the sliding along DNA, while the core domain suppresses the sliding [5]. p53 has four core domains, which can contact with DNA non-specifically [8]. We suggest that the sliding is promoted as the contact interaction decreases, and that p53 possesses loose contacts of the core domains to DNA in the fast mode and tight contacts in the slow mode. To confirm the above suggestion, we constructed the coreless mutant and examined its sliding dynamics (Fig. 7 and supplementary text). The coreless mutant showed a single sliding mode having the diffusion coefficient larger than the fast mode of the pseudo-WT p53, supporting our

suggestion that the different interactions between p53 and DNA involving the core domain are the origin of the two sliding modes.

The comparison of the diffusion coefficient for the two sliding modes of the pseudo-WT p53 and that for the coreless mutant suggests that the core domain may interact with DNA strongly in the slow mode and weakly in the fast mode. The multiple binding conformations of p53 in the p53-DNA complex have been suggested. For example, the electron microscopy studies demonstrate multiple quaternary structures of p53-DNA complex [41,42]. In addition, single-molecule FRET and NMR studies demonstrated that the core domain partly interacts with the C-terminal domain and the other core domains [43-45]. These interactions may regulate the conformational change of p53 on DNA. These results support our proposal that the loose and tight contacts of the core domains to DNA might be the origin of the multiple sliding modes of p53.

Oijen and his collaborators suggested the presence of a hypothetical two modes, search and recognition modes, in the target search process of p53 [5]. It was discussed that the search and recognition modes exchange each other in the time scale of about 1 ms, which is much shorter than the transition time between the fast and slow modes identified in this investigation (more than 330 ms). Thus, we are in the opinion that the fast and slow modes do not correspond to the search and recognition modes proposed by Oijen *et al* [5]. We suggest that the possible interactions between the core domains or between the core domain and the C-terminal domain in p53 might retard the transition between the two modes.

The multiple sliding modes were also observed in other DNA-binding proteins. A diverse diffusion coefficient in the 1D sliding was observed in LacI repressor, *E. coli* replisomes and DNA glycosylases [46-48]. A paused motion was detected in bacterial UvrA/UvrB complexes and DNA glycosylases [49,50]. These proteins cover a wide range of oligomeric states from a monomer to more than tetramer. The data suggest that even monomeric proteins have a potential to possess loose and tight contacts to DNA, resulting in different sliding modes. Furthermore, the presence of multiple binding sites to DNA in one protein, such as dimeric or oligomeric proteins, might be one of the factors to cause multiple sliding modes. Thus, the presence of the multiple sliding modes might be a general observation for DNA-binding proteins including p53 and play a pivotal role in their functions.

Physiological implications of the current observations

The concentrations of the divalent cations to alter the 1D sliding dynamics of p53 are in the range of the physiological concentrations. The total concentrations of Mg^{2+} and Ca^{2+} in nucleus/chromatin of a living cell were estimated to be 2-4 mM and 4-6 mM, respectively [51]. The total concentration of Mg^{2+} in mammalian cells ranges between 14 and 20 mM, and the concentration of free Mg^{2+} is 0.5-0.7 mM [52]. The concentration of free Ca^{2+} in the nucleus is 0.1-0.2 mM [53]. These results suggest that Mg^{2+} and Ca^{2+} could bind to DNA specifically in living cells, which might promote the movement of p53 along DNA.

The sliding dynamics of p53 might be regulated by the loss of homeostatic control for divalent cations. In apoptosis, the Ca^{2+} concentration is increased [11,12], which accelerates the sliding of p53 but reduces the binding time, and eventually leads to a slight increase in the sliding distance of p53 (Fig. 8). In contrast, the Mg^{2+} deficiency in cell cycle arrest suppresses the sliding of p53 but increases the binding time, and results in a small decrease of the sliding distance of p53 (Fig. 8). In both cases, the trade-off between the diffusion of the sliding and the binding time may not dramatically alter the search distance of p53 even in the loss of homeostatic control for divalent cations. However, the effect of divalent cations on the 3D search and/or the accuracy of the recognition should also regulate the efficiency and search time of p53 to the target sites. The current results demonstrating the effect of divalent cations to the 1D sliding call for the necessity of further investigations to understand the search mechanism of p53 relevant to physiological conditions.

Materials and Methods

Expression and purification of p53

The cDNA of human wild-type p53 and the artificially synthesized gene (Funakoshi, Co Ltd.) for the thermostable and cysteine-modified mutant of human p53, the pseudo-WT (C124A, C135V, C141V, W146Y, C182S, V203A, R209P, C229Y, H233Y, Y234F, N235K, Y236F, T253V, N268D, C275A, C277A, K292C) were inserted into a plasmid, pGEX-6P-1. The wild-type and the pseudo-WT possess additional sequences GPLGSASKL and GPLGS at the N-terminus, respectively. These proteins were expressed in BL21(DE3)pLysS *E. coli* cells. The cells were cultured in 1 L of 2×YT media at 37°C. After OD₆₀₀ of the culture reached 0.6, IPTG and ZnCl₂ were added both to the final concentration of 100 μM, and the cells were cultured at 20°C for 20 hours and harvested by centrifugation. The pellet was resuspended in the buffer containing 10-mM Na₂HPO₄, 1.8-mM KH₂PO₄, 2.7-mM KCl, 650-mM NaCl, 1-mM DTT, 10-mM MgCl₂, 1-mg/mL lysozyme, and 10-unit/mL DNase (pH 7.4), and lysed by sonication on ice. To remove DNA included in *E. coli*, 650-mM NaCl was used [54]. The lysed cells were further incubated on ice for 30 min. The supernatant was collected after centrifugation. GST-tagged p53 was purified by GST column (GSTrapTM FF, GE Healthcare, Tokyo, Japan). p53 was cleaved from the GST-tag by Precision protease (GE Healthcare, Tokyo, Japan) in the column. The sample was further purified by heparin column (HiTrapTM Heparin HP, GE Healthcare, Tokyo, Japan). The expression and purification of p53 were confirmed by SDS-PAGE. Western blot analysis for the wild-type p53 was further conducted

using two mouse monoclonal antibodies, p53(Pab240) and p53(DO-1) (Santa Cruz Biotechnology Inc, Texas, USA). The elution solution of p53 was stored at -80°C.

Fluorescence Anisotropy

Binding ability of p53 to DNA was quantitatively examined by using fluorescence anisotropy. Fluorometer (FP-6500, JASCO Co., Tokyo, Japan) coupled with automatic titration accessory (ATS-443, JASCO Co., Tokyo, Japan) and home-build autorotating polarizer unit was used. p53 was titrated in the quartz cuvette including a fluorescence-labelled DNA at the concentration of 5 nM in the buffer containing 20-mM HEPES, 50-mM KCl, 2-mM MgCl₂, 0.5-mM EDTA, 1-mM DTT and 0.5-mg BSA (pH 7.9) at 25 °C. The sequences of spDNA-FAM are 5'-6-FAM-ATCAGGAACATGTCCCAACATGTTGAGCTC-3' and 3'-TAGTCCTTGACAGGGTTGTACAACTCGAG-5' (Sigma-Aldrich Co., Tokyo, Japan). The underlined parts correspond to p21 promoter. The sequences of nspDNA-FAM are 5'-6-FAM-AATATGGTTTGAATAAAGAGTAAAGATTTG-3' and 3'-TTATACCAAACCTTATTTCTCATTCTAAAC-5' (Sigma-Aldrich Co., Tokyo, Japan), which was used for nonspecific binding. The excitation wavelength was 480 nm and the fluorescence of 525 nm was detected. A long-pass filter, which passes the wavelength longer than 500 nm, was used to prevent the scattered light from reaching the detector. All the measurements were finished within 50 min after the dilution of the stock solution to prevent the dissociation of the tetramer [27].

The observed data was analyzed by two-state binding model in which tetrameric p53 binds to DNA with a binding stoichiometry of 1:1. The observed anisotropy r_{obs} was fitted by using the equations:

$$r_{\text{obs}} = r_{\text{D}} \frac{(c_{\text{D}} - c_{\text{pD}})}{c_{\text{D}}} + r_{\text{pD}} \frac{c_{\text{pD}}}{c_{\text{D}}} \quad (3),$$

$$c_{\text{pD}} = \frac{(c_{\text{D}} + c_{\text{p}} + K_{\text{D}}) - \sqrt{(c_{\text{D}} + c_{\text{p}} + K_{\text{D}})^2 - 4c_{\text{D}}c_{\text{p}}}}{2} \quad (4),$$

where r_{D} , r_{pD} , K_{D} , c_{p} , c_{D} represent the anisotropy of free DNA, the anisotropy of p53-DNA complex, the apparent dissociation constant, the concentration of the tetrameric p53 and the concentration of DNA, respectively.

To measure the dissociation rate constant of p53 from nspDNA, we performed the stopped-flow kinetic fluorescence-anisotropy measurements by using a home-build system. An output from a 473-nm laser (Laser Quantum co., Ltd., Cheshire, UK) passed through a polarizer (Edmund Optics Japan Co., Ltd., Tokyo, Japan) was introduced into the flow cell of the stopped-flow apparatus (Unisoku Co., Ltd., Osaka, Japan). The fluorescence from FAM was detected by a photosensor (H10722, Hamamatsu Photonics, KK, Hamamatsu, Japan) after passing through a polarizer (Edmund Optics Japan Co., Ltd., Tokyo, Japan) and a band-pass filter (FF01-520/35, Semrock Inc., NY, USA). A mixture of p53 tetramer and nspDNA-FAM at the concentration of 60 nM and 40 nM, respectively, was mixed with a solution of spDNA with no fluorescent dye at the concentration of 400 nM both in

the buffer containing 20-mM HEPES, 50-mM KCl, 0-3-mM MgCl₂, 0.5-mM EDTA, 1-mM DTT and 0.5-mg BSA at 25 °C and at pH 7.9. The mixing ratio was 1:1. The dead time was 1 ms. The dissociation of p53 from DNA was detected as decrease of the fluorescence anisotropy caused by the transfer of p53 from the fluorescent nspDNA to the non-fluorescent spDNA. Thus, two types of fluorescence intensity data, I_{VV} and I_{VH} , were detected at the same condition, where I_{VV} stands for the fluorescence intensity excited at the vertical polarization and detected at the vertical polarization, and I_{VH} excited at the vertical polarization and detected at the horizontal polarization. Several traces of $I_{VV}(t)$ and $I_{VH}(t)$ were independently measured as a function of time t . The time resolution was 1 ms. The time course of fluorescence anisotropy, $r(t)$, was calculated from the averaged traces of $I_{VV}(t)$ and $I_{VH}(t)$ based on equation (5):

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)} \quad (5).$$

I_{HV} represents the fluorescence intensity excited at the horizontal polarization and detected at the vertical polarization, and I_{HH} excited at the horizontal polarization and detected at the horizontal polarization. The value of G factor, G , which was obtained from I_{HV} divided by I_{HH} , was 1. The trace of fluorescence anisotropy, $r(t)$, was then fitted with a single exponential, and the dissociation rate constant was obtained.

Labeling of fluorescent dye

The pseudo-WT p53 was labeled with ATTO532 (ATTO-TEC GmbH, Siegen, Germany) using a maleimide chemistry. p53-ATTO532 was purified using cation exchange column (HiTrapTM SP HP, GE Healthcare, Tokyo, Japan). The labeling ratio was determined as 1.4 dyes per a monomer of the mutant by observing the absorbances of 280 nm and 532 nm. The absorption coefficient of the p53 mutant is 30,350 M⁻¹cm⁻¹ at 280 nm. p53-ATTO532 was stored at -80°C.

Flow cell

The flow cell was constructed from a cover glass and a slide glass (Matsunami Glass Ind. Ltd., Osaka, Japan) [28]. The double-sided tape sealed the outer regions of the glasses to create the flow path between these glasses. Two holes were created in the slide glass using an electric drill before constructing the flow cell. One of the hole was connected to a syringe pump (μ-ton mini, Senshu Scientific co., Tokyo, Japan) through the PTFE tube whose inner diameter was 0.5 mm (GL Science, Tokyo, Japan), and the other hole was connected to a waste bottle. The connectors were purchased from Upchurch Scientific Inc. (Harbor, WA, USA). The flow cell was constructed from the slide glass and the coverslip passivated with PEG-biotin and PEG [29,30]. 50 mg/ mL of PEG-biotin (NANOCS Inc., MA, USA) and 50 mg/mL of PEG were used for the coating to tether one end of λDNA on the coverslip.

Biotinylated λ duplex DNA

Biotin-11-dGTP (PerkinElmer, Inc., MA, USA) was introduced into both ends of λ DNA (NEW ENGLAND BioLabs Inc, MA, USA) by the end-filling reaction with Klenow exo- (NEW ENGLAND BioLabs Inc., MA, USA).

TIRF microscopy

An output of 532-nm laser (CL532-050-L; CrystaLaser, NV, USA) was 10 times expanded and collimated on the back focal plane of an objective lens with $N.A.$ = 1.4 (Nikon, Tokyo, Japan) using a spherical lens (f = 500 mm). The flow cell was placed in front of the objective lens. The excitation light was introduced through the peripheral portion of the objective lens corresponding to $N.A.$ at around 1.40. The alignment enables to illuminate the sample with the total-internal reflection mode. The fluorescence was collected by the same objective, and was imaged by TDI-EM CCD camera (Hamamatsu Photonics, KK, Hamamatsu, Japan) after passing through the dichroic mirror and a spatial filter unit. The dichroic mirror (Di01-R405/488/532/635-25 $\times$ 36, Semrock Inc., NY, USA) was placed between the objective lens and the spherical lens so as to split the excitation light and the fluorescence from p53 on DNA. The spatial filter unit (Optonica Co., Kyoto, Japan) is composed of a slit and two multiple lenses (f = 200 and 70 mm). An optical filter (BLP01-532R-24, Semrock Inc., NY, USA) was mounted between the spatial filter unit and the camera. A spherical lens (f = 70 mm)

was used to focus the fluorescence on the camera. The final magnification of the image was 100. The laser power was 0.2 mW.

Single-molecule measurements

A neutravidin solution at 0.2 mg/mL in a buffer containing 25-mM Tris-HCl and 50-mM NaCl at pH 7.5 was introduced into the PEG-biotin-coated flow cell and incubated for 30 min to construct the neutravidin-coated flow cell [6,29,30]. To prevent non-specific binding of p53 to the surface, the blocking buffer containing 20-mM Tris-HCl, 50-mM NaCl, 2-mM EDTA, and 10-mg/mL BSA at pH 7.5 was introduced and incubated for 30 min. The biotinylated λ DNA in the buffer containing 20-mM Tris-HCl, 50-mM NaCl, 2-mM EDTA and 1-mg/mL BSA at pH 7.5 was then introduced into the neutravidin-coated flow cell at a concentration of 30 pM and incubated for 20-30 min to tether one end of DNA on the coverslip. Untethered DNA was then washed out by introducing the same solution. The number of the tethered DNAs was 5~20 in the field of view with a diameter of 30 μ m. The pseudo-WT p53 in the buffer containing 20-mM HEPES, 0.5-mM EDTA, 0-3-mM $MgCl_2$ or 0-1.5-mM $CaCl_2$, 0.5-mM DTT, 2-mM trolox, and 0.5-mg/mL BSA at pH 7.9 was introduced at the concentration of 0.5-2 nM at the rate of 500 μ L/min, and the single-molecule measurements were performed. The dilution of p53 from the stock solution at 10 μ M to the above solution at 0.5-2 nM was performed immediately before the experiments. All the measurements were finished within 50 min after the dilution of the stock solution to prevent the dissociation of the tetramer [27]. The

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5 concentrations of free Mg^{2+} and Ca^{2+} in the solution were estimated using a concentration-sensitive
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8 fluorescent dye, mag-fura-2. The inaccuracies of Mg^{2+} concentrations in our experiments were within
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17 **Analysis of single-molecule data**

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20 A software ImageJ and its plugin, Particle track and analysis, were used to track the center
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23 of the fluorescent spots based on 2D Gaussian fitting. The two-dimensional trajectories were rotated
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26 to adjust the longitudinal and traverse axes of the DNA to x and y axes, respectively. The positions of
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29 p53 were represented by x_i and y_i with the data point i . Firstly, the trajectories with more than 15 data
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32 points were selected. Secondly, we chose the trajectories which satisfy the following criteria to
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35 exclude the trajectories of p53 adsorbed on the coverslip. The local MSD of every segments with 10
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38 data points in the trajectories $\langle (x_{i+5} - x_i)^2 \rangle$ was calculated and the trajectories in which all the values of
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41 the local MSDs are larger than $0.0015 \mu\text{m}^2$. This threshold corresponds to 84% in cumulative
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44 distribution of the local MSDs in the absorbed molecules. Thirdly, we removed the trajectories whose
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47 $|x_{i+1} - x_i|$ is larger than 300 nm, because these are likely due to miss-connection between the
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57 **Acknowledgement**

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Figure legends

Figure 1. a) Static fluorescence anisotropy measurements for the titration of the pseudo-WT p53 against spDNA (red circle) and nspDNA (blue circle) labeled by FAM in the presence of 2 mM Mg^{2+} . The curves were the best-fitted curves obtained by using Eq. (3) and (4). b) Static fluorescence anisotropy measurements for the titration of the pseudo-WT p53 against nspDNA in the presence of 0~3-mM Mg^{2+} . Red, blue, green, orange and black circles were respectively the data in the presence of 0, 1, 1.5, 2, and 3-mM Mg^{2+} . The curves were the best-fitted curves. c) The apparent dissociation constant of the pseudo-WT p53 from nspDNA at the different concentrations of the divalent cations. Triangles and circles represent data obtained in the presence of Ca^{2+} and Mg^{2+} , respectively. The error bars represent the fitting error.

Figure 2. a) Kinetic fluorescence anisotropy measurements for the dissociation process of the pseudo-WT p53 from nspDNA labeled by FAM. Black traces from top to bottom were the data in the presence of 0, 1, 1.5, 2, and 3 mM Mg^{2+} , respectively. Red curves were the best-fitted curves with a single exponential. b) The apparent dissociation rate constant of the pseudo-WT p53 from nspDNA at the different concentrations of divalent cations. Triangles and circles represent data obtained in the presence of Ca^{2+} and Mg^{2+} , respectively. The error bars represent the fitting error.

Figure 3. a) Schematic representation of the set up for the single-molecule experiments. One end of λ DNA (black line) was labeled with biotin and attached on the coverslip of the flow cell via neutravidin (brown). The coverslip was coated with polyethylene glycol conjugated with biotin (blue). The TIRF illumination based on an oil immersion objective was used to observe the position of p53 labeled by ATTO532 along the DNA stretched by flow. b) Typical trajectories for the movement of the pseudo-WT p53 bound on DNA along the longitudinal (red) and transverse (blue) directions in the presence of 2-mM Mg^{2+} . The flow pressure was applied from a positive to a negative value of the longitudinal direction. Dotted lines represent trajectories along the longitudinal direction of the p53 mutant adsorbed on the surface of the flow cell. c) Time courses of the averaged MSD of the pseudo-WT p53 in the longitudinal (circles) and transverse (triangles) axes in the presence of 2-mM Mg^{2+} . Squares are the averaged MSD for the molecules adsorbed on the surface of the flow cell. The inset shows the same data in the expanded scale. The errors of the average are the standard error.

Figure 4. a) Time courses of the averaged MSD of the pseudo-WT p53 observed in the presence of 0~3-mM Mg^{2+} . The red, green, blue, orange and yellow circles are the averaged MSD values in the presence of 0, 1, 1.5, 2, and 3-mM Mg^{2+} , respectively. b) Time courses of the averaged MSD of the pseudo-WT p53 observed in the presence of 0~1.2-mM Ca^{2+} . The red, green and blue circles are the averaged MSD values in the presence of 0, 0.7, and 1.2-mM Ca^{2+} , respectively. (c) The averaged diffusion coefficients of the pseudo-WT p53 at the different concentrations of divalent cations.

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5 Triangles and circles represent data obtained in the presence of Ca^{2+} and Mg^{2+} , respectively. The
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7 errors of the average are the standard error.
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14 Figure 5. The Mg^{2+} -concentration dependence of the displacement distributions at the time interval of
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17 165 ms. Red, green, blue, orange and yellow distributions are respectively calculated from the data of
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20 the pseudo-WT p53 in the presence of 0, 1, 1.5, 2 and 3-mM Mg^{2+} . Black curves represent the best
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23 fitted curves obtained by using Eq. (1). Dashed curves are the distributions of each mode.
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30 Figure 6. The Mg^{2+} -concentration dependence of the apparent diffusion coefficients (a) and the drift
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33 velocities (b) for the fast (circles) and slow (squares) modes of the pseudo-WT p53 obtained by the
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36 fitting of the displacement distributions at the time interval of 165 ms shown in Fig. 5. The error bars
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39 are the fitting errors.
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45 Figure 7. Comparison of the displacement distributions observed for the coreless mutant (a) and the
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48 pseud WT (b) of p53 at the time interval of 165 ms in the presence of 2-mM Mg^{2+} . Black curves
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51 represent the best-fitted curves obtained by using Eq. (2) for panel (a) or Eq. (1) for panel (b). Dashed
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54 curves in panel (b) are the distributions of each mode.
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Figure 8. The sliding distance expected for the pseudo-WT p53 after a single binding event to nspDNA. Triangles and circles represent data obtained in the presence of different concentrations of Ca^{2+} and Mg^{2+} , respectively.

Figures
Figure 1

Figure 2

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Figure 4

Figure 5

Figure 6

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Figure 7

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Figure 8