Supplementary information

Specific Conformational Dynamics and Expansion Underpin a Multi-Step Mechanism for Specific Binding of p27 with Cdk2/Cyclin A

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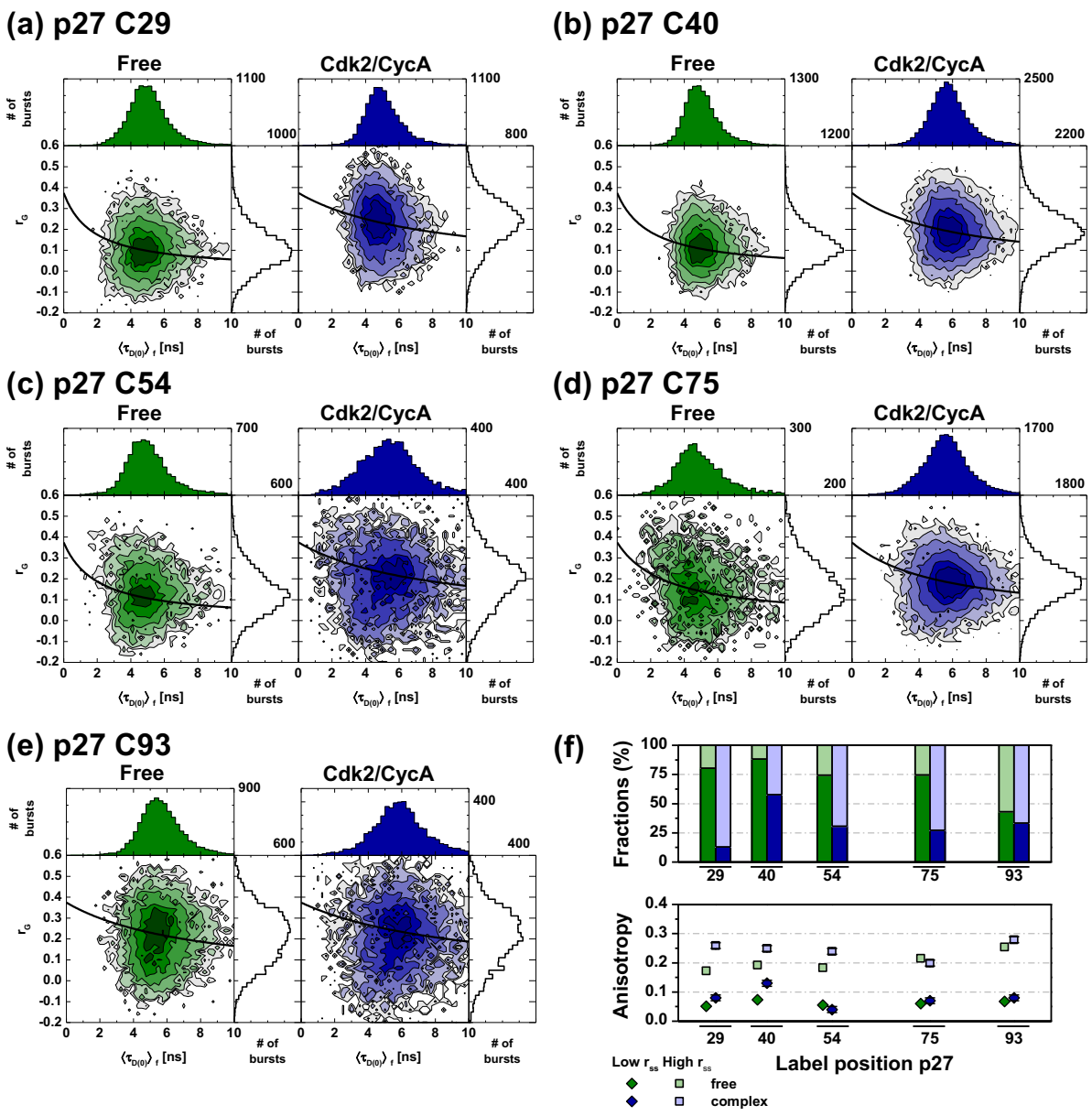

Figure S1. smFA 2D histograms of p27 single cystein variants free and in complex with Cdk2/Cyclin A. (a-f) The average fluorescence lifetime ($\langle\tau_{D(A)}\rangle_f$) of the BODIPY label on the x-axis vs. scatter-corrected anisotropy (r_D) on the y-axis are shown. One-dimensional frequency histograms along the axis and number of bursts at the right top corner are shown for each. In black, the Perrin equation is added through the center of the population to guide the eye. (f) Anisotropy and respective fractions for p27 free and bound to Cdk2/Cyclin A give information about local dynamics for the molecule based on global fits using PDA for three time windows (Δt =1, 2, and 3 ms) . The corresponding fractions and anisotropy values (r_s) for the Low and High r_s are presented in dark and light colors, respectively. Fit results are collected in Table S1.

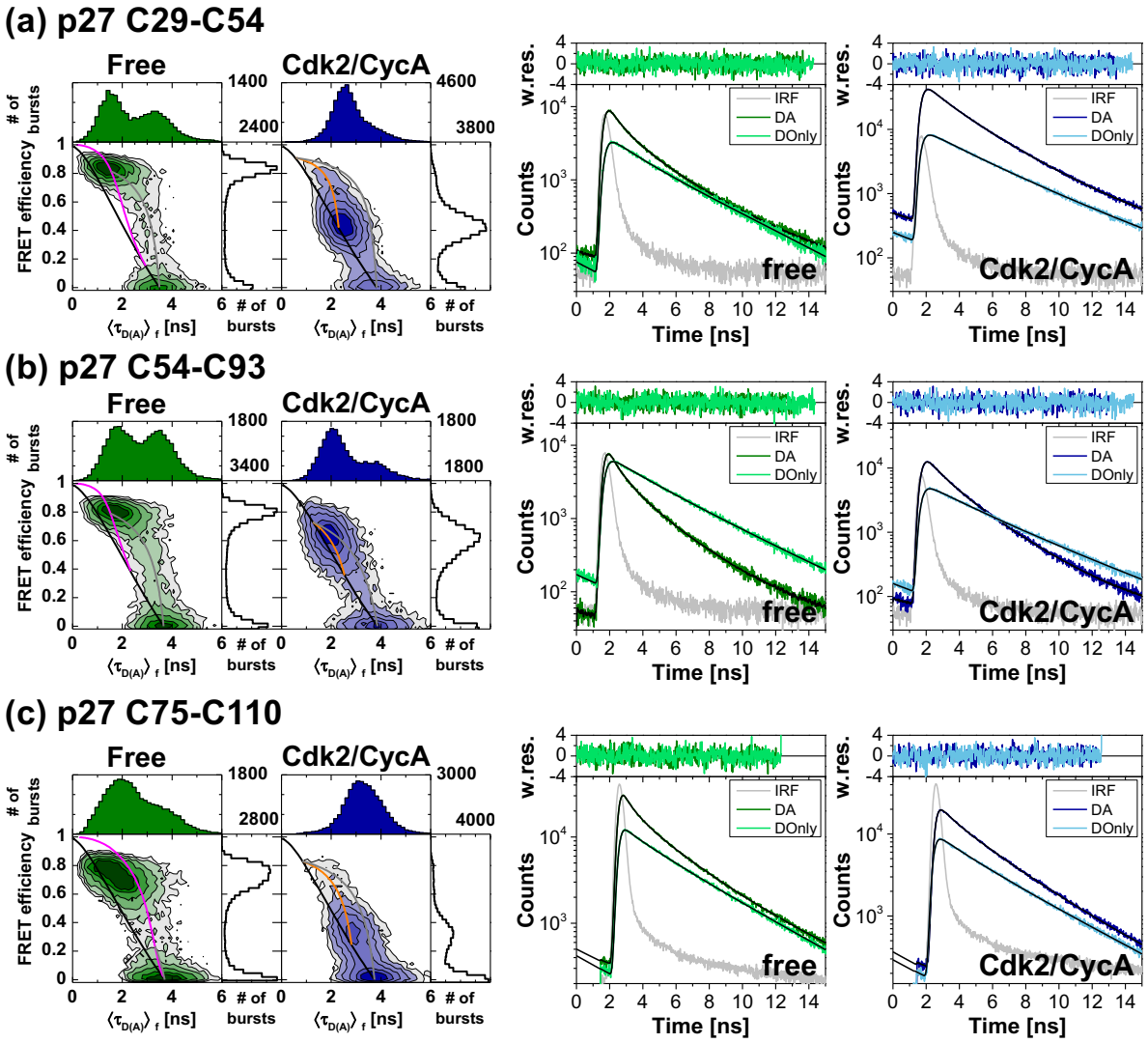

Figure S2. smFRET efficiency and sub-ensemble fluorescence decays for p27 double cysteine variants free and in complex with Cdk2/Cyclin A. (Left) The 2D histogram shows that the FRET efficiency *E* is decreasing from free (green) to the complex-bound form (blue). The average fluorescence lifetime ($\langle\tau_{D(A)}\rangle_f$) are shown on the x-axis and FRET efficiency *E* on the y-axis (left). For each FRET indicator, 1D frequency histograms along the axis and number of bursts at the right top corner are shown. In black the static FRET line and in grey the bleaching/folding line for both conditions is shown, while for the free p27 we added the dynamic WLC line (magenta) and for the Cdk2/Cyclin A bound p27 the dynamic Gaussian distances line (orange). (Middle, Right) Time resolved fluorescence decays for extracted DA (dark green/blue) and DOnly populations (light green/blue) are shown. Instrument response function (IRF) is shown in grey. Weighted residuals for the respective fit model (multiexponential for DOnly, WLC for free p27 and Gaussian distances for bound p27) are shown on top. All burst from the 2D histograms below *E* of ~ 0.18 were considered to belong to the DOnly population, i.e. either missing an active acceptor and/or a too far D-A distance to allow for FRET to happen. Details see Methods and Supporting Methods, fit results are summarized in Table S2.

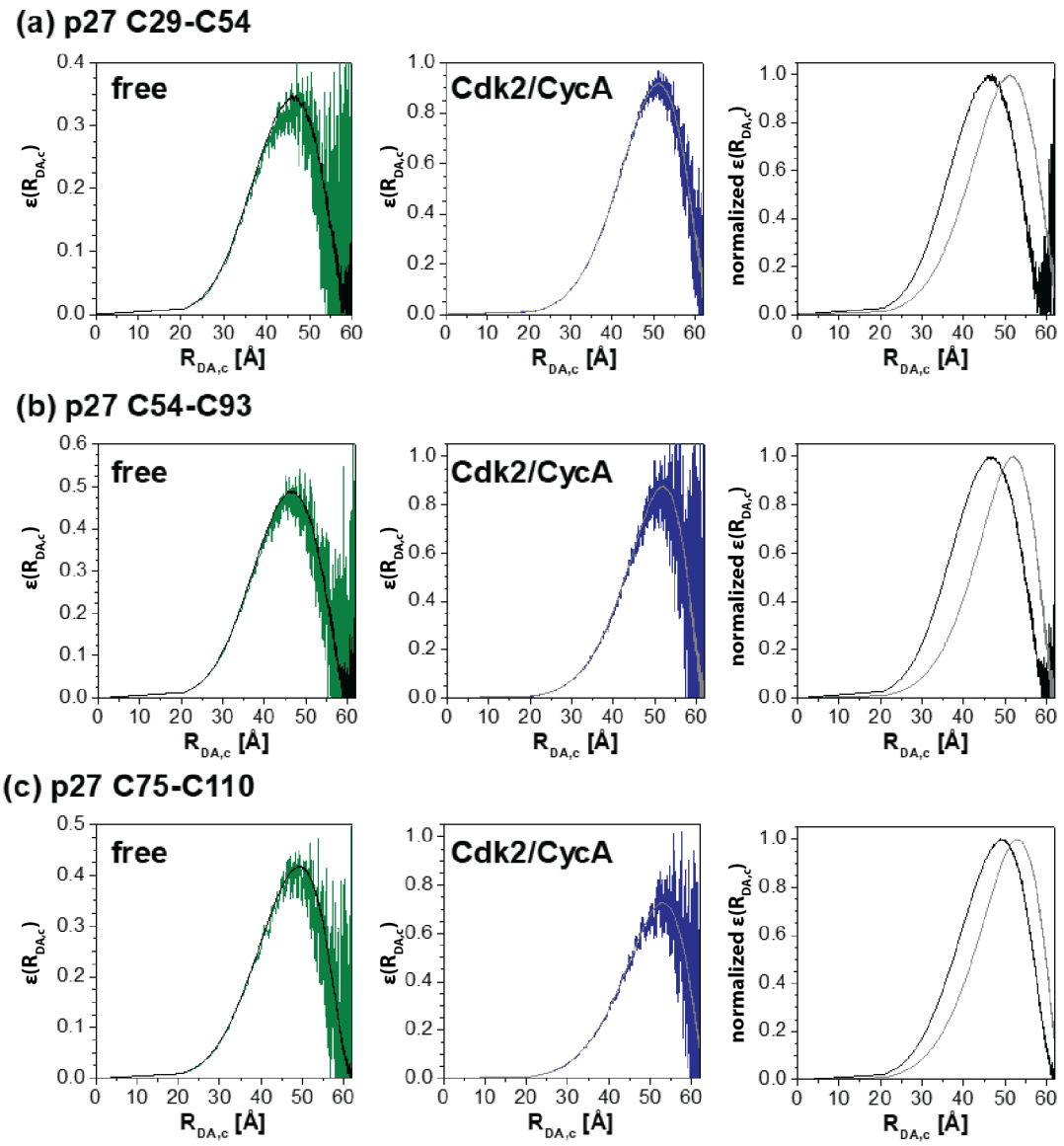

Figure S3. FRET-induced donor decays ε(t) for seTCSPC analysis. (Left) WLC fit of DA-population, in green the raw data and in black the fit is shown. (Middle) Fit of two Gaussian distributed distances for p27 in complex with Cdk2/Cyclin A, in blue the raw data and in light grey the fit is shown. (Right) Normalized ε(t) for free (black) and bound (light grey) p27 for easier comparison. All p27 FRET variants show a shift to longer distances when bound to Cdk2/Cyclin A and a decreased width in distribution. Fit results are summarized in Table S2.

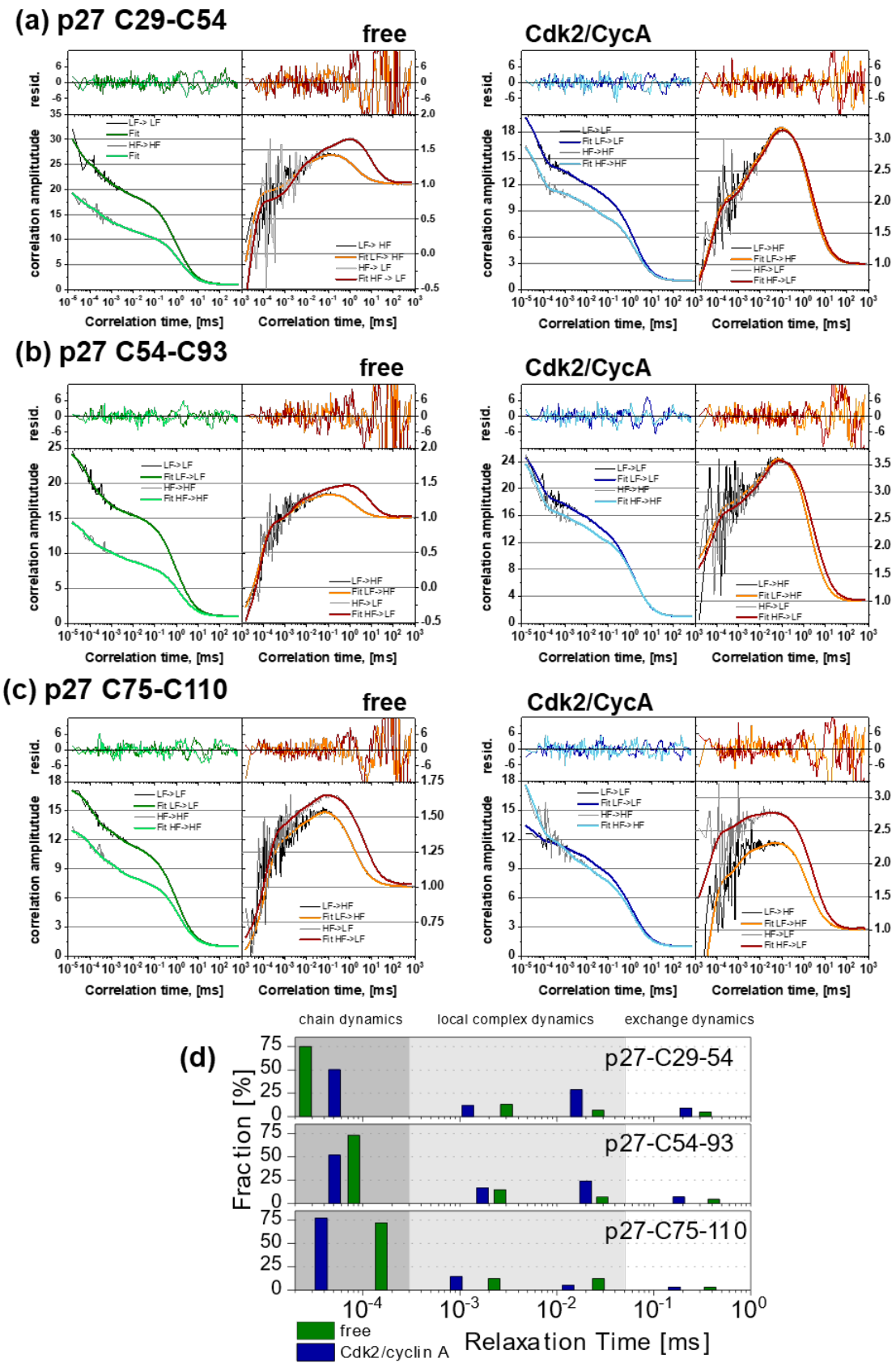
(a) p27 C29-C54
free
Cdk2/CycA
(b) p27 C54-C93
free
Cdk2/CycA
(c) p27 C75-C110
free
Cdk2/CycA
resid.
correlation amplitude
Correlation time, [ms]
LF-> LF
Fit
HF->HF
Fit
LF-> HF
Fit LF-> HF
HF-> LF
Fit HF -> LF
LF->LF
Fit LF->LF
HF->HF
Fit HF->HF
LF->HF
Fit LF->HF
HF->LF
Fit HF->LF
(d)
chain dynamics
local complex dynamics
exchange dynamics
p27-C29-54
p27-C54-93
p27-C75-110
Fraction [%]
Relaxation Time [ms]
free
Cdk2/cyclin A

Figure S4. Filtered FCS species-specific auto and cross correlation functions of smFRET for p27 double cysteine variants free and bound to Cdk2/Cyclin A. Filtered FCS auto (left panels) and cross (right panel) correlations for a) p27 C29-C54, b) p27 C54-C93 and c) p27 C75-C110. Filters were selected by "burstwise" selection based on the FRET efficiency arbitrary cutoffs to select for low-FRET (LF) or high-FRET (HF) populations. The integrated fluorescence of these bursts corresponds to two independent species. The two sCCF (right, LF- > HF and HF->LF) and the sACF (LF->LF and HF->HF) where fitted globally as described in [1]. Residuals of the fits for sACF and sCCF are shown at the top of each correlation curve. (d) Fractions of the relaxation times. Fit results are summarized in Table S3.

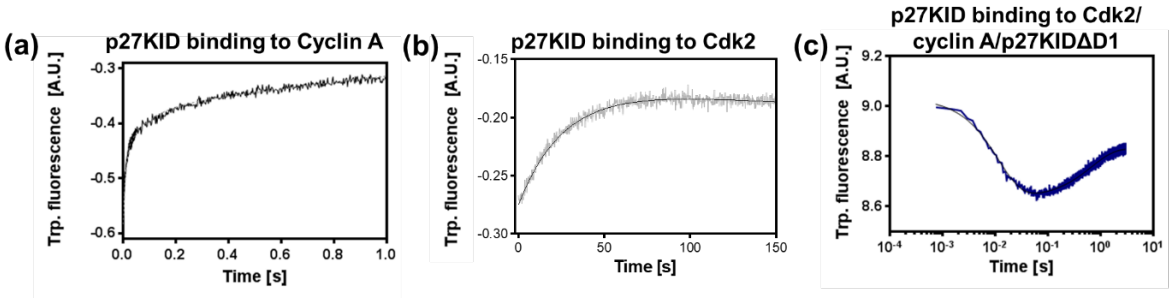

Figure S5. Tryptophan fluorescence during binding experiments for the interaction of p27KID with Cyclin A, Cdk2, and in ternary complex of Cdk2/Cycline A/p27KIDΔD1. a) Intrinsic tryptophan fluorescence increases with a very fast phase upon binding of p27KID to Cyclin A, followed by a slower phase, indicating fast initial binding followed by internal configurational changes. b) Fluorescence increases mono-exponentially for the interaction of p27KID with Cdk2. c) Tryptophan fluorescence shows a rapid decrease and a slow increase in the binding traces upon the interaction of p27KID with Cdk2/cyclinA/p27KIDΔD1 complex.

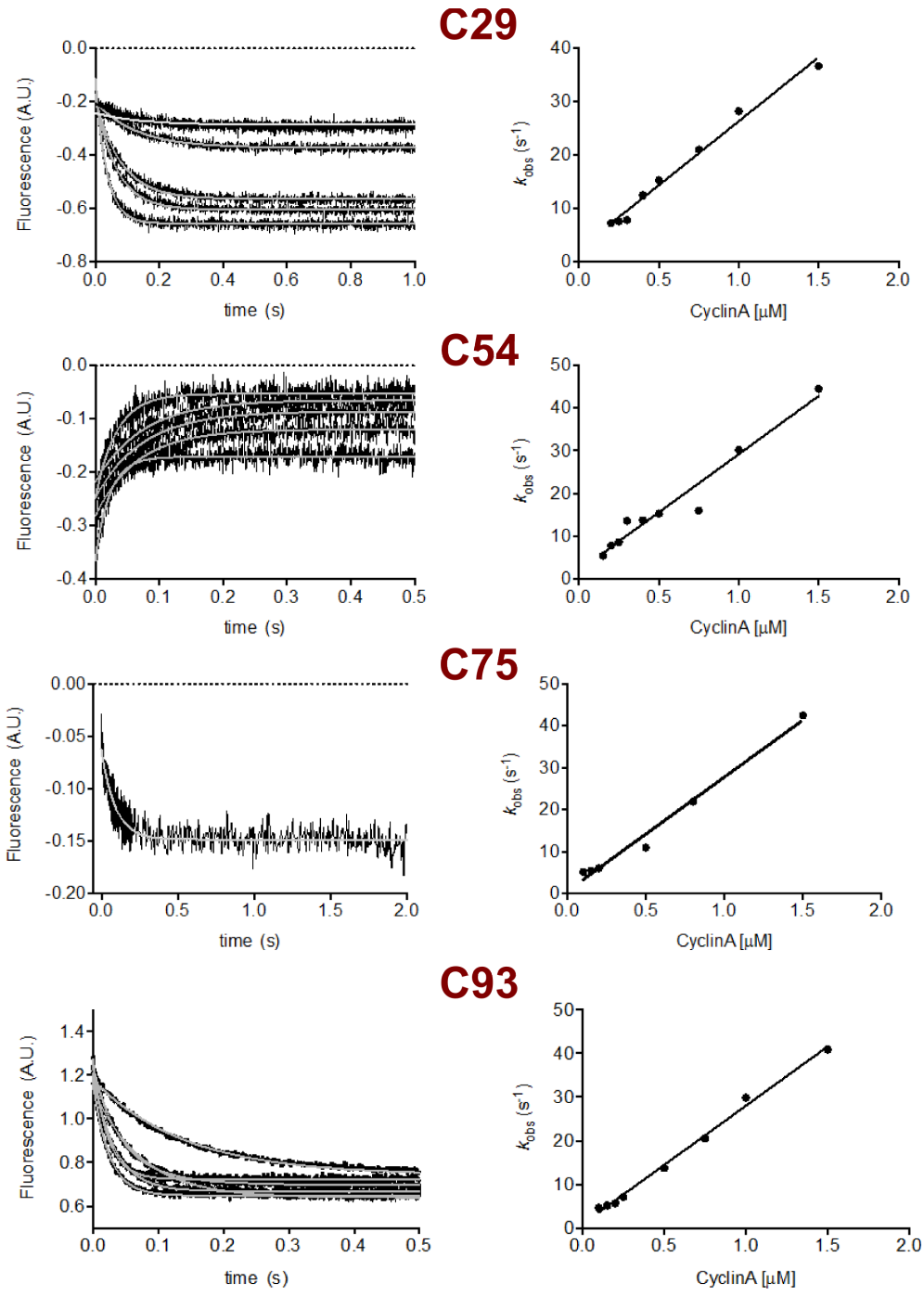

Figure S6. The association reactions and kinetics for the interaction of fluorescently labeled p27KID with Cyclin A. For these experiments, we labeled residues in and near the KID region of p27 with BODIPY-FL, namely sites C29, C40, C54, C75, and C93, mutated to cysteines (Fig. 1). Although the C54, C75 and C93 sites are not in the binding region of Cyclin A, the kinetic reaction shows a significant change in the fluorescence upon binding. The increased fluorescence for C54 in contrast to the other positions represents a different structural arrangement in the LH domain of p27 KID.

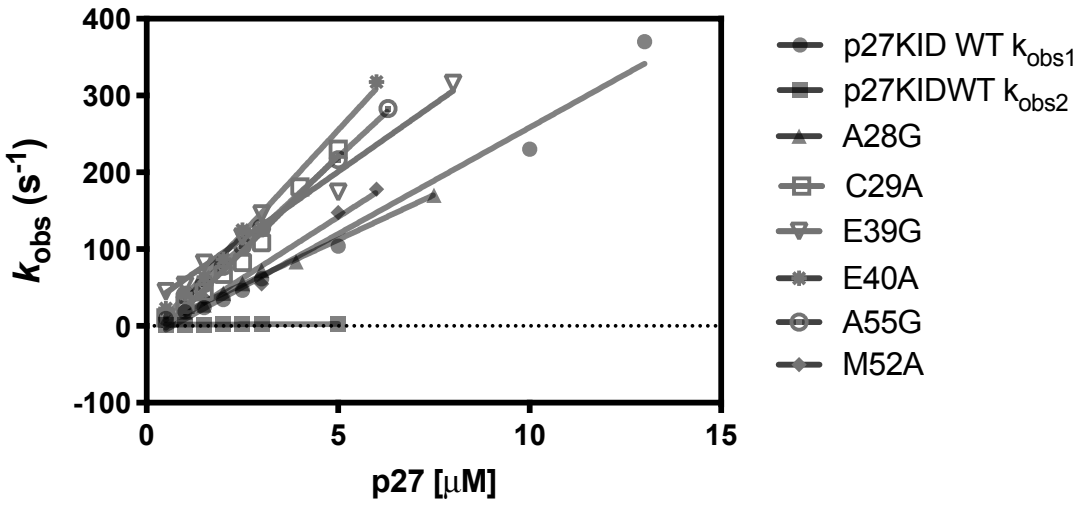

Figure S7. Binding kinetics study for the initial interaction of p27 with cyclin A is performed for different mutants of p27 by tryptophan fluorescence. The mutants in p27, A28G, C29A & E39G (located in D1) and E40A, M52A & A55G (located in LH), do not show any significant effect on the first, very fast or the second, slow rate constants.

Table S1. smFA fit parameters for free p27and bound to Cdk2/Cyclin A.

a) Fit parameter for two state model. Uncertainties were estimated by calculating the χ_r^2 distribution resulting from variation of each fit parameter and using twice the standard deviation as the uncertainty to establish a 95 % confidence interval. Parameters were varied by sampling a normal distribution centered at the mean (fit) values given in the table.

Residue number	free p27 Anisotropy (Fraction (%))		χ_r^2	p27 Cdk2/Cyclin A Anisotropy (Fraction (%))		χ_r^2
	Low r_G	High r_G		Low r_G	High r_G	
29	0.05±0.004 (80.5±4.0)	0.17±0.010 (19.5±16.4)	1.12	0.08±0.008 (12.9±10.8)	0.26±0.002 (87.1±1.6)	1.26
40	0.07±0.002 (88.3±2.7)	0.19±0.014 (11.7±20.4)	1.22	0.13±0.006 (57.7±6.9)	0.25±0.006 (42.3±9.5)	1.54
54	0.06±0.004 (74.5±4.8)	0.18±0.010 (25.5±14.1)	1.32	0.04±0.008 (30.8±8.4)	0.23±0.004 (69.2±4.9)	1.45
75	0.06±0.0006 (74.6±5.1)	0.22±0.012 (25.4±14.9)	0.94	0.07±0.010 (27.2±13.2)	0.20±0.004 (72.8±4.9)	1.26
93	0.07±0.004 (43.1±4.2)	0.26±0.004 (56.9±3.2)	1.68	0.08±0.008 (33.3±7.8)	0.28±0.004 (66.7±3.9)	1.18

b) Comparison of average Anisotropy: ($\langle r_G \rangle = b_1 r_{G,Low} + b_2 r_{G,High}$) and average rotational correlation time ($\langle \rho_D \rangle = b_1 \rho_{G,Low} + b_2 \rho_{G,High}$), where $\rho_{G,Low}$ and $\rho_{G,High}$ have been calculated by the Perrin equation ($r_0/r_x = 1 + \tau_G/\rho_{G,x}$). The overall tumbing of the observed molecule is reflected by $\langle r_G \rangle$. For a larger molecule, $\langle r_G \rangle$ is larger. Uncertainties were propagated from a) according to the propagation rule $\sigma_x^2 = \sum_i (\frac{dx}{dy_i})^2 \sigma_{y_i}^2$.

Samples	Free		Cdk2/Cyclin A	
	$\langle r_G \rangle$	$\langle \rho_G \rangle$ [ns]	$\langle r_G \rangle$	$\langle \rho_G \rangle$ [ns]
p27 C29	0.07±0.028	1.44±0.75	0.24±0.010	9.31±0.360
p27 C40	0.09±0.039	1.64±1.17	0.18±0.026	6.45±1.150
p27 C54	0.09±0.026	1.93±0.70	0.18±0.012	7.05±0.530
p27 C75	0.10±0.033	2.77±1.17	0.16±0.014	5.20±0.410
p27 C93	0.17±0.009	8.11±0.54	0.21±0.013	11.51±0.93
Average	0.10	3.18	0.19	7.90
stdev	0.02	0.87	0.02	0.68

Table S2. seTCSPC fit parameters for free samples.

a) Fluorescence lifetime fit for DOnly-labeled molecules free in solution

Sample free	τ_1 [ns]	x_1	τ_2 [ns]	x_2	τ_3 [ns]	x_3	$\langle\tau_f\rangle$ [ns]	$\langle\tau_x\rangle$ [ns]	χ_r^2
p27 C29 C54	3.80	0.669	1.21	0.331			3.45	2.94	0.99
p27 C54 C93	3.75	0.762	1.21	0.238			3.52	3.15	1.08
p27 C75 C110	4.00	0.626	2.43	0.132	1.33	0.242	3.66	2.89	1.09

b) Worm-like chain model for DA-labeled molecules free in solution

Sample free	*Length** [Å]	*persistence** [Å]	*flexibility***	x_{DOnly}	χ_r^2
p27 C29 C54	73.8 [62.0-104.0]	29.0 [19.0-40.7]	0.393	0.145	1.08
p27 C54 C93	65.7 [62.8-69.4]	30.4 [28.9-31.9]	0.463	0.060	1.14
p27 C75 C110	67.7 [63.0-73.0]	41.4 [36.0-49.0]	0.612	0.268	1.21

*values in brackets show show 95 % confidence interval

**flexibility is defined as the ratio of persistence length divided by total length

b) Maximum and width of FRET-induced donor decay

Sample	free		Cdk2/Cyclin A complex	
	Maximum [Å]	width* [Å]	Maximum [Å]	width* [Å]
p27 C29 C54	46.8	19.0	51.2	18.5
p27 C54 C93	46.9	18.5	52.1	15.8
p27 C75 C110	48.9	19.0	53.1	17.0

*width is determined at 50 % of the maximal height of the converted FRET-induced donor decay curve

Table S3.

a) Relaxation rate constants obtained from filteredFCS fit for free p27 and bound to Cdk/Cyclin A**.** Data was analysed as described previously [1].

Correlation		*b*	N_{CC}/N_{Br}	t_{diff}[ms]	ω_0/z_0	*CC*	t_{R1} [µs]	X_1/AC_1	t_{R2} [µs]	X_2/AC_2	t_{R3} [µs]	X_3/AC_3	t_{R4} [µs]	X_4/AC_4	*B*	t_B [ms]
p27 C29 C54																
Cdk2/ Cyclin A	LF-HF	1.01	0.37	1.76	3.70	1.24	0.058	0.52	1.96	0.17	22.7	0.24	207	0.07	0.10	4.03
	HF-LF	1.01	0.29			1.27									0.30	
	LF-LF	1.01	0.049					0.35		0.078		0.066		0.047	0.10	
	HF-HF	1.00	0.060					0.37		0.062		0.10		0.023	0.00	
free	LF-HF	1.00	1.86	1.36	6.93	4.94	0.022	0.75	2.63	0.13	23.1	0.069	297	0.049	0.16	5.12
	HF-LF	1.00	0.30			5.72									0.04	
	LF-LF	0.99	0.030					0.23		0.080		0.038		0.073	0.01	
	HF-HF	0.97	0.049					0.18		0.087		0.069		0.005	0.81	
p27 C54 C93																
Cdk2/ Cyclin A	LF-HF	1.00	0.34	1.77	3.42	0.82	0.058	0.50	1.39	0.12	18.3	0.29	247	0.09	0.01	5.76
	HF-LF	1.02	0.20			0.89									0.44	
	LF-LF	1.01	0.039					0.30		0.075		0.086		0.084	0.10	
	HF-HF	1.01	0.041					0.35		0.065		0.096		0.033	0.05	
free	LF-HF	1.01	2.29	1.29	4.68	4.60	0.070	0.73	2.27	0.15	25.7	0.07	363	0.05	0.12	4.96
	HF-LF	1.00	0.43			4.66									0.00	
	LF-LF	1.00	0.04					0.22		0.05		0.03		0.08	0.00	
	HF-HF	1.01	0.07					0.25		0.10		0.07		0.00	0.79	

p27 C75 C110															
Cdk2/ Cyclin A	LF-HF	1.00	0.69	1.52	4.05	2.30	0.043	0.77	1.06	0.14	15.1	0.05	188	0.04	0.00	2.53
	HF-LF	1.01	0.35			0.96									0.35	
	LF-LF	1.01	0.076					0.17		0.072		0.11		0.10	0.03	
	HF-HF	1.00	0.053					0.39		0.12		0.088		0.049	0.10	
free	LF-HF	1.01	1.67	1.29	6.71	1.91	0.13	0.72	1.96	0.12	23.4	0.12	331	0.04	0.00	3.80
	HF-LF	1.02	0.57			1.68									0.61	
	LF-LF	1.01	0.06					0.24		0.11		0.05		0.07	0.13	
	HF-HF	1.01	0.08					0.24		0.16		0.06		0.01	0.06	

b) Errors calculated for a). Errors were calculated by simultaneously sampling all parameters from normal distributions centered at each fit value, calculating the χ^2 distribution for all sampled paramer values, and using the F-test for the ratio of sampled curve χ^2 values to the χ^2 value for the curves corresponding to the value in a) to define the 95% confidence interval.

Correlation		*b*	N_{CC}/N_{Br}	t_{diff}[ms]	ω_0/z_0	*CC*	t_{R1} [µs]	X_1/AC_1	t_{R2} [µs]	X_2/AC_2	t_{R3} [µs]	X_3/AC_3	t_{R4} [µs]	X_4/AC_4	*B*	t_B [ms]
p27 C29 C54																
Cdk2/ Cyclin A	LF-HF	0.18	0.20	0.23	0.47	0.11	0.04	0.03	0.82	0.10	3.24	0.14	49.65	0.01	0.03	1.02
	HF-LF	0.09	0.11			0.22									0.19	
	LF-LF	0.06	0.01					0.01		0.01		0.01		0.02	0.01	
	HF-HF	0.01	0.02					0.01		0.01		0.01		0.02	0.00	
free	LF-HF	0.03	0.71	0.30	0.54	0.47	0.03	0.21	0.28	0.05	2.78	0.01	92.76	0.02	0.06	0.79
	HF-LF	0.24	0.09			0.52									0.01	
	LF-LF	0.35	0.07					0.01		0.03		0.03		0.01	0.01	
	HF-HF	0.01	0.02					0.01		0.01		0.05		0.01	0.09	

p27 C54 C93															
Cdk2/ Cyclin A	LF-HF	0.08	0.10	.16	0.70	.013	0.04	0.24	0.61	0.05	2.93	0.17	52.10	0.06	0.01	1.44
	HF-LF	0.08	0.04			0.33									0.02	
	LF-LF	0.01	0.01					0.01		0.01		0.01		0.01	0.02	
	HF-HF	0.01	0.01					0.01		0.01		0.01		0.01	0.02	
free	LF-HF	0.05	0.08	0.51	3.02	0.23	0.04	0.03	0.44	0.11	5.60	0.02	23.87	0.01	0.03	1.76
	HF-LF	0.07	0.08			0.17									0.19	
	LF-LF	0.26	0.01					0.06				0.03		0.08	0.38	
	HF-HF	0.24	0.01					0.11				0.07		0.01	0.32	
p27 C75 C110															
Cdk2/ Cyclin A	LF-HF	0.41	0.02	0.19	0.98	0.29	0.03	0.17	0.60	0.02	5.55	0.02	54.45	0.01	0.01	0.98
	HF-LF	0.43	0.13			0.89									0.19	
	LF-LF	0.03	0.01					0.01		0.01		0.01		0.02	0.02	
	HF-HF	0.06	0.01					0.01		0.01		0.01		0.01	0.01	
free	LF-HF	0.10	0.92	0.22	1.09	0.28	0.03	0.36	0.67	0.03	6.39	0.03	81.26	0.03	0.01	1.79
	HF-LF	0.06	0.48			0.44									0.60	
	LF-LF	0.39	0.01					0.13		0.08		0.02		0.04	0.13	
	HF-HF	0.48	0.01					0.01		0.01		0.01		0.01	0.06	

Table S4. Radii of hydration (determined from smFRETmeasurements) and gyration.

	29-54	54-93	75-110	Simulation
R_h* [Å]	18.86	17.90	17.90	
R_g [Å]				36.28

*Values for R_h calculated according to $R_h=4k_B\ \tau_D\ T/(6\pi\eta\omega^2)$, with T=298.15 K, τ_D the diffusion time for each sample, η=0.85 mPa*s, and ω^2=73900 nm^2 the square of the width of the confocal volume, determined by measuring the diffusion time of a standard sample with known diffusion time (Rhodamine-110, D = $4.7x10^{-6}$ cm^2/s).\

Table S5. Kinetic parameters determined by rapid mixing experiments.

Interacting molecules	K_a[$M^{-1}s^{-1}$]
p27KID + Cyclin A	$5.0x10^7$
p27KID + Cdk2	$1.0x10^{-2}$
p27KID + Cdk2/Cyclin A	$3.5x10^7$
p27KIDΔD1 + Cyclin A	-
p27KIDΔD1 + Cdk2	$1.5x10^{-2}$
p27KIDΔD1 + Cdk2/Cyclin A	$5.0x10^{-2}$
p27KID + Cdk2/Cyclin A /p27KIDΔD1	$1.6x10^7$

Table S6. Multiple Gaussian Fits for R_g.

Number of Gaussians	Means [Å]	Amplitudes	Standard Deviation [Å]	*p*-Value
1	34.8	1	15.9	0.85
2	28.1, 43.5	0.58, 0.42	7.1, 10.6	0.91
3	23.8, 30.0, 44.2	0.22, 0.44, 0.34	3.6, 6.4, 9.8	0.97

Table S7 Correction parameters for smFA and smFRET experiments

Correction parameters are calculated and implemented as described in [2]

Sample	Correction Parameter	Value
All samples		
	Ratio of green vs. red detection efficiencies (g_G/g_R)	0.8
	Ratio of green perpendicular vs. parallel detection efficiencies (G_g)	1.069
	Spectral cross-talk, α	1.7 %
	Direct acceptor exchitation, β	1.3 %
	Optical element depolarization factor, l_1	0.0176
	Optical element depolarization factor, l_2	0.0526
All free single-labeled BODIPY for smFA		
	Mean green channel background signal, B_G	0.55 kHz

All complex single-labeled BODIPY for smFA		
	Mean green channel background signal, B_G	1.07 kHz
p27-C29-C54 free		
	Mean green channel background signal, B_G	1.62 kHz
	Mean red channel background signal, B_R	1.03 kHz
	Donor fluorophore quantum yield, $\Phi_{F,D}$	0.61
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.46
p27-C54-C93 free		
	Mean green channel background signal, B_G	1.62 kHz
	Mean red channel background signal, B_R	1.03 kHz
	Donor fluorophore quantum yield, Φ_D	0.66
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.46
p27-C75-C110 free		
	Mean green channel background signal, B_G	0.65 kHz
	Mean red channel background signal, B_R	0.42 kHz
	Donor fluorophore quantum yield, $\Phi_{F,D}$	0.69
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.42
p27-C29-C54 + Cdk2/Cyclin A		
	Mean green channel background signal, B_G	1.57 kHz
	Mean red channel background signal, B_R	0.94 kHz
	Donor fluorophore quantum yield, $\Phi_{F,D}$	0.61
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.46
p27-C54-C93 + Cdk2/Cyclin A		
	Mean green channel background signal, B_G	1.57 kHz
	Mean red channel background signal, B_R	0.94 kHz
	Donor fluorophore quantum yield, $\Phi_{F,D}$	0.61
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.47
p27-C75-C110 + Cdk2/Cyclin A		
	Mean green channel background signal, B_G	0.65 kHz
	Mean red channel background signal, B_R	0.42 kHz
	Donor fluorophore quantum yield, $\Phi_{F,D}$	0.69
	Acceptor fluorophore quantum yield, $\Phi_{F,A}$	0.42

Movie S1

The supplementary movie represents ~65 ns of the total simulation time. It shows various aspects of the p27 dynamics on different timescales, including transient formation and loss of LH helical secondary structure, bunching and elongation of the post-KID region near the helix, and global compaction and elongation.

Supplementary References

[1] Tsytlonok M, Sanabria H, Wang Y, Felekyan S, Hemmen K, Phillips AH, et al., Dynamic anticipation by Cdk2/Cyclin A-bound p27 mediates signal integration in cell cycle regulation, Nature communications. 10 (2019) 1676.
[2] Hellenkamp B, Schmid S, Doroshenko O, Opanasyuk O, Kuhnemuth R, Rezaei Adariani S, et al., Precision and accuracy of single-molecule FRET measurements-a multi-laboratory benchmark study, Nat Methods. 15 (2018) 669-676.