Supplementary Information: Material and Methods

Quantitative analysis of FLIM data

The fluorescence lifetime images and histograms of figure 5A show apparent fluorescent lifetimes that depend on the relative concentration of complex present, the spectroscopic properties of the donor and acceptor fluorophores, and on the binding geometry of the complex (relative distance and orientation). A decrease in apparent fluorescence lifetimes is a robust measure to detect FRET and assess binding. To obtain more quantitative information about the relative concentration of complexes and the binding geometry, the FLIM data were further analysed using global analysis (Verveer et al., 2000). This analysis assumes that the donor-tagged molecule exists in two states, each of which has a unique fluorescence lifetime. One state is that of the donor-tagged molecule alone, where the fluorescence lifetime (τ_D) is equal to that of the donor in the absence of FRET. The other state is that of the complex, where the fluorescence lifetime of the donor (τ_{DA}) is reduced by energy transfer to the acceptor fluorophore. It is assumed that the fluorescence decay of the donor alone is adequately described by a single-exponential decay, yielding a single value for τ_D that is invariant throughout the cell. Furthermore, it is assumed that the geometry of binding in the complex is relatively fixed, so that the rate of energy transfer and therefore τ_{DA} is also invariant. These conditions are generally well met when GFP is used as the donor and for tight binding of a single acceptor-tagged molecule to the donor-tagged molecule. By simultaneously analysing multiple FLIM data sets, it is then possible to calculate the two invariant lifetime values τ_D, and τ_{DA}, from which the energy transfer efficiency within a single complex can be determined by $E = 1 - \tau_{DA}/\tau_D$. Since the energy transfer efficiency is determined by the distance and orientation of donor and acceptor fluorophores in the complex, a change in E gives an indication for a change in binding geometry in a complex. In the case of Cdt1GFP, the energy transfer efficiency was calculated to be very similar when the wild type or mutant forms of Geminin were used as FRET acceptors (55.9% and 56.6% respectively). Therefore, the reduced FRET in the presence of mutant Geminin can be attributed to a reduced affinity of Cdt1 for the mutant (Δ90-120) Geminin and not to a change in orientation or distance within the complex. In addition, in each pixel of the image, the relative concentration of donor-tagged molecules in complex with respect to the total concentration of donor-tagged molecules can be calculated. For the case of the Cdt1 and Geminin this means that in each pixel the relative concentration of the Cdt1-Geminin complex [Cdt1-Geminin]/[Cdt1] can be calculated.

Analysis of *in vivo* binding isotherms

For a binding reaction that follows the laws of mass-action it is expected that the concentration of Cdt1-Geminin complexes will depend on the expression level of the components Cdt1 and Geminin. Thus a plot of the fraction of bound Cdt1 as a function of Geminin intensity should show increased values for increasing expression levels of Geminin. To show that this is the case we derive a formula for the relative concentration of complex as a function of Geminin concentration from the law of mass-action. First we define:

[C] : total concentration of Cdt1.
[G] : total concentration of Geminin.
[CG] : concentration of Cdt1-Geminin complex.
[C_f] : concentration of free Cdt1.

$[G_f]$: concentration of free Geminin.

Then the total concentrations of Cdt1 and Geminin are related to the concentrations of free molecules and of the complex by:

$$[C] = [C_f] + [CG] \text{ and } [G] = [G_f] + [CG].$$

The law of mass action states that:

$$[CG] = K[C_f][G_f] = K([C] - [CG])([G] - [CG]),$$

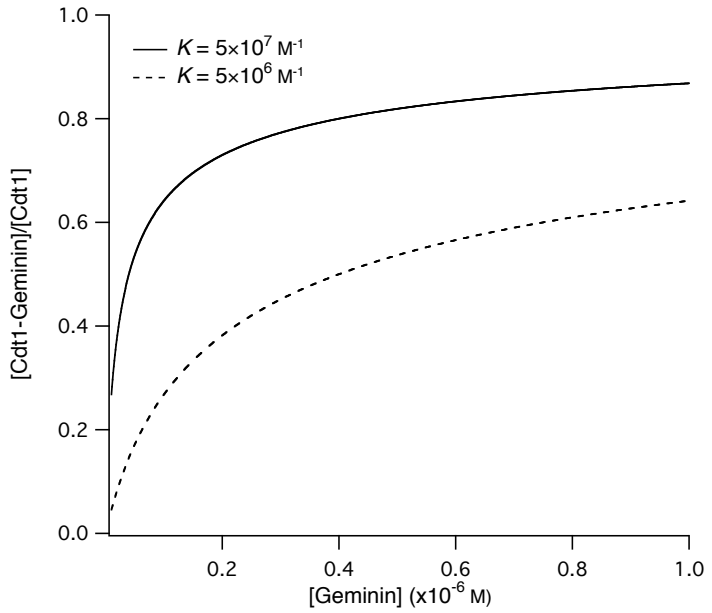
where $K = K_{on}/K_{off}$ is the equilibrium constant. Solving for [CG] and dividing by [C] gives two solutions for the relative fraction of bound Cdt1-Geminin:

$$\frac{[CG]}{[C]} = \frac{1 + K[C] + K[G] \pm \sqrt{(1 + K([C] + [G]))^2 - 4K^2[C][G]}}{2\,K[C]}.$$

Since the bound fraction should be zero if [G] equals zero, the physical solution corresponds to the minus sign. To simplify the analysis of the solution, we observe that the intensities of Cdt1 and Geminin show a linear correlation in the FLIM data (not shown), and we therefore substitute $[C] = R[G]$, where R is a constant. We then obtain:

$$\frac{[CG]}{[C]} = \frac{1 + (1 + R)K[G] - \sqrt{(1 + ((1 + R)K[G]))^2 - 4RK^2[G]^2}}{2\,RK[G]}.$$

It can be proven that [CG]/[C] is a monotonically increasing function that approaches zero at [G] = 0. Moreover, its derivative with respect to K can be proven to be always positive for all physical values of R and [G], showing that if the equilibrium constant K decreases, the curve of [CG]/[C] shifts downwards. This behaviour is illustrated in the plot below, where the effect of a ten-fold decrease of K is shown:



The binding isotherms of Figure 5B follow a behaviour similar to that depicted above, showing that they follow the law of mass-action, as one would expect. Moreover, the clear downward shift of the binding curve of Cdt1 to mutant Geminin shows that the equilibration constant is substantially lower compared to binding to wt Geminin, demonstrating the lower affinity of mutant Geminin. Thus, *in vivo* binding isotherms of this type can be used to assess changes in affinity. The equations presented here could in principle be fit to the data, but this requires knowledge of the absolute concentrations of Geminin, and of Cdt1 or *R*. Although the measured fluorescence intensities are proportional to the concentrations, their calibration to true concentrations is non-trivial and not feasible with current FLIM measurements. Therefore, the actual values of the equilibrium constant *K*, or a fold-change of *K*, cannot be calculated from these data.

Cell culture and cell transfection

MCF7 cells were grown in DMEM/high glucose with 10% (v/v) fetal bovine serum. For FRAP experiments, MCF7 cells were synchronized in S phase by a 22 hour incubation with 2.5 mM thymidine, released for 4 hours into G2 and synchronized for 8 hours in mitosis by incubation with 1mg/ml nocodazole. FACS analysis showed that following the treatment, 75% of cells were arrested in mitosis. For chromatin fractionation experiments, cells were synchronized in mitosis as above and chromatin fractionations performed as previously described (Nishitani et al., 2004).

Antibodies, Immunofluorescence and Western blotting

For Western blotting, total cell lysates were prepared by lysing cell pellets directly in SDS-PAGE loading buffer. Affinity antibodies against hCdt1 were described previously (Nishitani et al., 2001). Immunofluorescence on MCF7 cells and stable cell lines, using affinity purified anti-Cdt1 (1:150), anti-cyclin A (1:40, Neomarkers), anti-414 (1:1000 kindly provided by I.Mattaj) and anti-GFP (1:200 Molecular Probes) was carried out as previously described (Nishitani et al., 2001). Quantifications were performed with IPLab software (Scanalytics Inc.Fairtax,VA,USA).

Plasmid constructs

To construct fusions of Cdt1 to GFP and dhcRed, full-length Cdt1 (Nishitani et al., 2001) was subcloned into pcDNA3.1/EGFP (Invitrogen) between the AflII and KpnI sites and into pdiHcRed-N1 (kindly provided by J. Ellenberg) between the NheI and KpnI sites by introducing appropriate sites by polymerase chain reaction. pcDNA3.1nlsGFP was generated by introducing 3 copies of the SV40 Nuclear Localization Sequence and a single myc epitope between the KpnI and BamHI sites of pcDNA3.1/EGFP. The mutant form Cdt1Δ1-140 was subcloned from pCMV-hCdt1(141-end)NLSmyc (Nishitani et al., 2004) into pcDNA3.1nlsGFP and pdiHcRed-N1. Cdt1Δ173-450 was made by removing the internal Eco47III fragment from pcDNA3.1Cdt1GFP. Plasmids Cdt1Δ150-170GFP and Cdt1Δ170-190GFP were constructed by replacing internal Cdt1 fragments in pcDNA3.1Cdt1GFP with fragments bearing the deletions (kindly provided by K. Helin (Ballabeni et al., 2004)). Cdt1Δ298-352 was generated by PCR and inserted into pcDNA3.1nlsGFP. Cdt1-Cy, Cdt1 A6 and Cdt1 A6-Cy have been previously described (Nishitani et al., 2006) and were subcloned in pcDNA3.1nlsGFP. Mutants Δ1-140, Δ298-352 and Cdt1Cy, which failed to correctly localize to the nucleus, were fused to three copies of the SV40 nuclear localization sequence and the myc epitope at the C-terminus of the molecule,

before the GFP. Full-length hGeminin was subcloned into pcDNA3.1/EGFP, into pcDNA3.1nlsGFP, and into pdiHcRed-N1 by inserting appropriate sites by PCR. GeminΔ90-120 (kindly provided by K. Helin (Ballabeni et al., 2004)) was subcloned into pdiHcRed-N1.

References

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