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ORIGINAL PAPER

Acceptor-photobleaching FRET analysis of core kinetochore and NAC proteins in living human cells

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Abstract Faithful chromatin segregation is mediated and controlled by the kinetochore protein network which assembles at centromeres. In this study, the neighbourhood relations of inner kinetochore and nucleosome-associated complex (NAC) proteins were analysed in living human interphase cells by acceptor photobleaching FRET. The data indicate that CENP-U is in close vicinity to CENP-I as well as to CENP-B and that CENP-M is close to CENP-T.

Keywords Centromere · Kinetochore · Mitosis · Live-cell imaging · NAC complex · Acceptor-photobleaching FRET

Introduction

Centromeric chromatin consists of interspersed regions in which H3 histones are replaced by CENH3 (in humans: CENP-A; for reviews see Allshire and Karpen 2008; Black and Bassett 2008). This centromeric chromatin region is framed by pericentromeric heterochromatin. During interphase, centromeres form a specialised chromatin of a compact structure (interphase pre-kinetochore) distinct from the trilaminar structure of the kinetochores in mitosis after nuclear envelope break down (Roos 1973; Marshall et al. 2008). The kinetochore is a multi-protein complex which assembles at centromeres and guides correct DNA segregation. The kinetochores contain a structural core including the nucleosome-associated complex (NAC) and CENP-A distal complex (CAD) proteins, termed the constitutive centromere-associated network (CCAN), and further proteins and protein complexes, including KNL-1, Hec 1 and hMis12 complexes (for reviews, see Cheeseman and Desai 2008; Musacchio and Salmon 2007). The NAC proteins (CENP-C, -H, -U, -M, -T and -N) were purified in association with CENP-A nucleosomes, while the CAD proteins could only be purified with NAC proteins (Foltz et al. 2006; Okada et al. 2006). CENP-T and CENP-W form a DNA-binding complex which directly associates with nucleosomal DNA and with histone H3 (Hori et al. 2008a). Also proteins of the FACT (Foltz et al. 2006) and the RSF complex (Perpelescu et al. 2009) bind to CENP-A chromatin. Here we analyse the core kinetochore and NAC proteins including CENP-I.

The detailed functions of the CCAN proteins (Hori et al. 2008a) are unclear; their presence at centromeres, however, is required for correct kinetochore function. Depletion of these proteins in human or chicken cells causes chromosome congression and mitotic defects (Liu et al. 2003; Minoshima et al. 2005; Foltz et al. 2006; Okada et al. 2006). siRNA down-regulation of CENP-M, CENP-N or CENP-T caused an increase in the number of cells in mitosis (Foltz et al. 2006). The organisation of the inner kinetochore is dynamic: some inner kinetochore proteins show a cell-cycle-dependent assembly which provides both stability by sustained binding of some components (CENP-A and CENP-I) and differing degrees of flexibility through dynamic exchange of other components (i.e. CENP-B, CENP-H, CENP-C) (Hemmerich et al. 2008; Hellwig et al. 2008).

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The CCAN proteins can be grouped into different functional classes (Okada et al. 2006; McClelland et al. 2007; Hori et al. 2008b). The in vivo analysis of CCAN proteins, selected according to their functional classes, will be subject of future studies. Here we used another criterion for selection: since the kinetochore proteins CENP-M, -N and -U can be affinity purified with tagged CENP-A (Foltz et al. 2006), they are expected to be located in the kinetochore complex in close proximity to one another (Hori et al. 2008a). This view is supported by siRNA protein knockdown experiments (Foltz et al. 2006; Okada et al. 2006; Hori et al. 2008a). Here we therefore set out to measure the direct proximity between the inner kinetochore proteins CENP-A, -B, -C, -H, -I, -M, -T and -U in vivo by acceptor-photobleaching fluorescence resonance energy transfer (FRET) in living human cells.

Materials and experimental methods

Plasmids

Plasmids pDF149, pDF152, pDF153 and pDF197 encoding LAP-CENP-M, -N, -T and -U fusion proteins were used for amplification of full-length CENP-M, -N, -T and -U by PCR (Expand high fidelity^{PLUS} PCR System, Roche, Penzberg, Germany) applying forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGCGCCACCATGGGCATGTCGGTGTTGAGGCCCCTG-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGGTCCTCCAGGGAGGGGC-3′ for Cenp-M, forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGCGCCACCATGGATGAGACTGTTGCTGAGT-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATCTCTAATTTTAAAATAATTCATTCTC-3′ for Cenp-N, forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGCGCCACCATGGCTGACCACAACCCTGAC-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGGCAGGGAAGACAGAGTT-3′ for Cenp-T, and forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGCGCCACCATGGGCACTAGTATGGCCCCGCGGG-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCCCTGGTCAAGGAGCTTCTCTAA-3′ for Cenp-U. CENP-M, -N, -U and -T harbouring linear PCR fragments were transferred into vector pDONR221 by BP recombination reaction (Invitrogen, Carlsbad, CA, USA). After verification by sequencing (MWG Biotech, Ebersberg, München, Germany), the genes were cloned by LR recombination reactions into various modified pEFP-C- and pEFP-N-based destination vectors (BD Biosciences, Clontech, Palo Alto, CA, USA). As a result, we obtained expression vectors carrying the genes coding for CENP-M, -N, -T and -U fused to the C termini of EGFP, Cerulean, YFP, and mCherry. In the constructed fluorescent proteins (FP)-CENP-M, -N, -T and -U, the amino acid (aa) linker between the fused proteins is SGTSLYKKAGFENLYFQGAT. Due to the cloning protocol, the aa sequence TQLSCTKW is added to the C-terminal ends of FP-CENP-M, -N, -T, and -U.

For FRET analysis with EGFP and mCherry as donor-acceptor pair, we constructed various mCherry-Cenp fusions. pEYFP-C1-Cenp-A, pEYFP-C2-CENP-B (Orthaus et al. 2008) and pEGFP-C2-Cenp-C (Hemmerich et al. 2008) were digested with AgeI and BsrGI, and for all three vectors, the 722-bp fragments were replaced with the 713-bp AgeI-BsrGI mCherry-harbouring fragment from pmCherry-H2A, resulting in pmCherry-C1-Cenp-A, pmCherry-C2-Cenp-B and pmCherry-C2-Cenp-C. pEGFP-C1-CENP-A resulted from the replacement of EYFP with EGFP in *Age*I–*Bsr*GI-digested pEYFP-C1-CENP-A (Orthaus et al. 2008). For construction of pEYFP-C2-CenpI, the 722-bp *Age*I-*Bsr*GI fragment of pEGFP-C2-Cenp-I (Hemmerich et al. 2008) was replaced with the 722-bp EYFP-carrying fragment of pEYFP-C2. A vector expressing the fusion pEGFP-mCherry served as a reference vector for positive FRET control measurements. The linker sequence between the N-terminal EGFP and mCherry is SGLRSRGDPAT.

All clones were verified by sequencing (MWG Biotech, Ebersberg, Germany). Full-length protein expression of the fusion constructs was confirmed by Western blots.

Cell culture and transfection into HEp-2 cells

HEp-2 (HeLa contaminant) cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (PAA Laboratories) in a 9.5% CO_2 atmosphere at 37°C and grown to subconfluency as recommended. At this stage, the medium was removed and cells were washed with magnesium- and calcium-containing PBS (Sigma-Aldrich, Taufkirchen, Germany) followed by detachment with trypsin/EDTA (PAA Laboratories).

The detached cells were dissolved in fresh DMEM and re-seeded in new culture dishes. For live-cell imaging experiments, cells were seeded on 42-mm glass dishes (Saur Laborbedarf, Reutlingen, Germany) 1 or 2 days before experiments and transfected with plasmid DNA 24–48 h before observation using FuGENE HD Transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Alternatively, the DMEM-dissolved cells were counted in an improved Neubauer cell (Superior Marienfeld, Lauda-Königshofen, Germany). Aliquots of 10^6 cells were centrifuged for 10 min at 1,600 rpm. The medium was removed, and cells were suspended in 100 µl Nucleofector solution V (Amaxa, Walkersville, MD, USA) and mixed with plasmid DNA. After electroporation with the Nucleofector (Amaxa) transfected cells were seeded on 42-mm glass dishes (Saur Laborbedarf) 1 or 2 days before live-cell imaging experiments.

Western blots

To control the full-length protein expression of the fusion constructs, Western blots were carried out as described previously (Orthaus et al. 2008). In short, transfected HEp-2 cells were taken from culture flasks, counted in a Neubauer chamber and lysed for 10 min at 100°C in an appropriate volume of 2% SDS, 0.1% bromphenole blue, 35 mM dithiothreitole, 25% glycerol and 60 mM Tris–HCl pH 6.8. Cell lysates with appropriate amounts of total protein were separated by SDS-PAGE and blotted onto Protran BA nitrocellulose (Schleicher and Schuell, Dassel, Germany). Proteins reacted with mouse monoclonal antibody against EGFP (#sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:50. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (#115-035-072, Jackson Immunoresearch Laboratories, West Grove, PA, USA) at a dilution of 1:4,000 and finally with the ECL-advance system (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The chemiluminescence was detected by Biomax light-1 Kodak film (Kodak, Stuttgart, Germany).

Acceptor-photobleaching-based FRET measurements

When FRET occurs, both the intensity and lifetime of the donor fluorescence decrease while the intensity of the acceptor emission increases. All these changes can be exploited to measure the efficiency of energy transfer between the donor and the acceptor (Nagy et al. 1998; Chen et al. 2003; Jares-Erijman and Jovin 2003, 2006; Elder et al. 2009). Two FRET pairs were studied: EGFP-mCherry and Cerulean-EYFP.

Cerulean fluorescence was excited with the argon 458-nm laser line and detected using the Meta detector (ChS1 + ChS2: 477–499 nm). EYFP fluorescence was excited with the argon 514-nm laser line and detected in one of the confocal channels using a 530-nm long-pass filter. EGFP fluorescence was excited with the argon 488-nm laser line and detected using the Meta detector (ChS1 + ChS2: 505–550 nm). mCherry fluorescence was excited with the argon 561-nm laser line and detected in one of the confocal channels using a 575-nm long-pass filter. To minimise cross-talk between the channels, each image was collected separately in the multi-track mode, i.e. both fluorophores were excited and recorded specifically and separately. Single optical sections were selected by scanning the sample in the *z*-axis for optimal fluorescence signals.

Acceptor photobleaching was achieved by scanning a region of interest (ROI) including one centromere of a nucleus 100 times (scans at 1.6-µs pixel time) using the 514-nm (EYFP) and 561-nm (mCherry) laser line at 100% intensity. Bleaching times per pixel were identical for each experiment. However, total bleaching times differed depending on the size of the bleached ROIs and the used magnification. Three donor and acceptor fluorescence images were taken before and 7 to 12 images were taken after the acceptor-photobleaching procedure to assess changes in donor and acceptor fluorescence. To minimise the effect of photobleaching of the donor during the imaging process, the image acquisition was performed at low laser intensities. To compare the time course of different experiments, donor intensities in the ROI were averaged and normalised to the intensity measured at the first time point after photobleaching, and acceptor intensities in the ROI were averaged and normalised to the mean intensity measured at the three time points before photobleaching.

Results

The proteins closely associated with centromeric nucleosomes can be purified by CENP-A pull-down (Foltz et al. 2006; Okada et al. 2006) and thus are expected to be located in the direct neighbourhood of one another in the kinetochore complex (Hori et al. 2008a). These proteins localise to centromeres in interphase (Foltz et al. 2006). We confirmed this localisation in interphase HEp2 cells (Hellwig et al. 2008; data not shown). For FRET analyses in living human cells, these proteins were cloned in fusion with several FP (two FRET pairs: Cerulean-EYFP and EGFP-mCherry). Full-length expression of each fusion protein was verified by Western blot analysis (Hemmerich et al. 2008; Orthaus et al. 2008, 2009; data not shown).

When the fluorescent tag is fused to CENP-H via a peptide linker of 11 to 16 amino acids, CENP-H centromere localisation is prevented irrespective of whether the tag is fused to the N or the C terminus. However, a 39 amino acids long linker between CENP-H and the fluorescent protein (placed at the N terminus of CENP-H) reestablished centromere localisation (Hemmerich et al. 2008) and enabled dynamic studies. However, the long linker might place the fluorescent tag too far away from CENP-H for

FRET results to be interpreted on a molecular basis. Therefore, CENP-H was not studied by FRET.

For the other kinetochore proteins studied here, the fluorescent tag did not detectably alter the properties of the proteins in terms of their proper localisation and centromere-binding characteristics. Upon expression in HEp-2 cells, fluorescently tagged inner kinetochore proteins localised in vivo to centromeric regions, reflecting the distribution of endogenous centromere proteins (Hemmerich et al. 2008; Orthaus et al. 2008, 2009; data not shown). EGFP-tagged CENP-A was previously shown to be correctly incorporated into nucleosomes of centromeric heterochromatin (Wieland et al. 2004) indicating that the fluorescent tag did not detectably influence the localisation of the fusion protein. CENP-B-EGFP and EGFP-CENP-C tightly bound to human centromeres (Hemmerich et al. 2008) indicating that these tagged kinetochore proteins, similar to tagged CENP-A (Foltz et al. 2006), also retained functional properties of the endogenous proteins.

Human HEp-2 cells were co-transfected with two vectors containing two different genes each coding for a kinetochore protein fused to a fluorescent protein (having different colours, i.e. a FRET pair) for acceptor photobleaching FRET measurements (Wouters et al. 1998). In this approach, the donor fluorescence intensity is measured (in the same sample) in the presence of an acceptor before and after destroying the acceptor by photobleaching (Kenworthy 2001); potential energy transfer between the donor and the acceptor is eliminated by irreversibly photobleaching the acceptor, resulting in an increase in the donor fluorescence (Wouters et al. 1998; Kenworthy and Edidin 1998; Berney and Danuser 2003). As FRET pairs, we used Cerulean-EYFP and EGFP-mCherry as donor and acceptor fluorophores (Orthaus et al. 2008; Tramier et al. 2006). Fluorescence intensity changes in the bleached area were recorded over time in the donor and acceptor channels by sequential imaging scans. FRET measurements were carried out in several independent experiments.

FRET control experiments were carried out as presented previously (Orthaus et al. 2008, 2009). In short, as a positive control, after acceptor photobleaching, the triple fusion ECFP–EYFP–CENP-A showed strong FRET between the neighbouring fluorescence tags. Other control experiments excluded effects due to photobleaching (Patterson et al. 1997) or photo-conversion (Creemers et al. 1999; Malvezzi-Campeggi et al. 2001). Furthermore, no negative influence of the photobleaching procedure on cell morphology was observed (data not shown; Orthaus et al. 2008). The analysis of non-fused donor and acceptor fluorophores, co-transfected in living human cells, did not result in FRET so that we can exclude any false-positive FRET due to an unspecific association of the fluorescent tags (Orthaus et al. 2008). Thus, if energy transfer was observed between the labelled proteins, it could only be due to a specific (direct or indirect) interaction between the proteins but not between the tags.

We then analysed in living human HEp-2 cells whether the N termini of the human kinetochore proteins CENP-M, -N, -T and -U are close to the N termini of CENP-A, -B, -C, -I, -N, -T, -U and -M at human centromeres. The data were collected and treated in the following way. In each image, two centromere locations were identified (marked "1" and "2" in Fig. 1). Only at spot 2 was the acceptor bleached so that in case of FRET, the donor fluorescence intensity increased. At spot 1, the acceptor is not bleached; this location served as control. Any influence of the environment during the experiment or any influence of the acceptor photobleaching at another location in the sample on the donor might influence the donor fluorescence intensity. These 'background' FRET signals were recorded at spot 1 by measuring the time course of the donor fluorescence intensity before and after photobleaching. The donor fluorescence intensity at spot 2 was also changed due to these influences, but also due to FRET.

The donor fluorescence intensities at the bleached (spot 2) as well as the non-bleached (spot 1) locations were recorded and treated equally: at spot 2 the FRET efficiency was calculated according to $E_{\mathrm{FRET}} = 1 - (I_{\mathrm{DA}}/I_{\mathrm{D}})$ where I_{DA} and I_{D} are the donor fluorescence intensities in the presence and absence of the (intact) acceptor respectively. I_{DA} was obtained by averaging the donor intensities of the three pre-bleached images in the presence of a photochemically intact acceptor. I_{D} was determined by measuring the donor fluorescence intensity in the first image obtained after the acceptor was destroyed by photobleaching. At the unbleached spot 1, the donor fluorescence change was calculated according to $E_{\mathrm{var}} = 1 - (I_{\mathrm{DAbefore}}/I_{\mathrm{DAafter}})$ so that a corresponding value for variation efficiency, E_{var}, is obtained, which can directly be compared to the FRET efficiency E_{FRET}. The E values were grouped into intervals of 4%, and the number of cases (y-axis) was plotted versus E values (x-axis) with the x-axis values as the mid-point of the range (0–4%: mid-point 2, 4–8%: mid-point 6%, etc.) resulting in distribution diagrams of both E_{FRET} and E_{var}. E_{var} indicates the donor fluorescence intensity variation during the experiment due to background effects. FRET was interpreted to have occurred when E_{FRET} was clearly larger than E_{var}.

CENP-U showed a structured presence within the whole-cell nucleus and co-localised with CENP-A at centromeres (Fig. 1a, left pictures). Two centromeric locations were selected, spot 1 and spot 2 indicated in Fig. 1a and enlarged in Fig. 1b. Figure 1a and b display the fluorescence of the fusion proteins at both locations before and after photobleaching of the acceptor. Then, the acceptor mCherry fused to CENP-A was nearly completely photo-

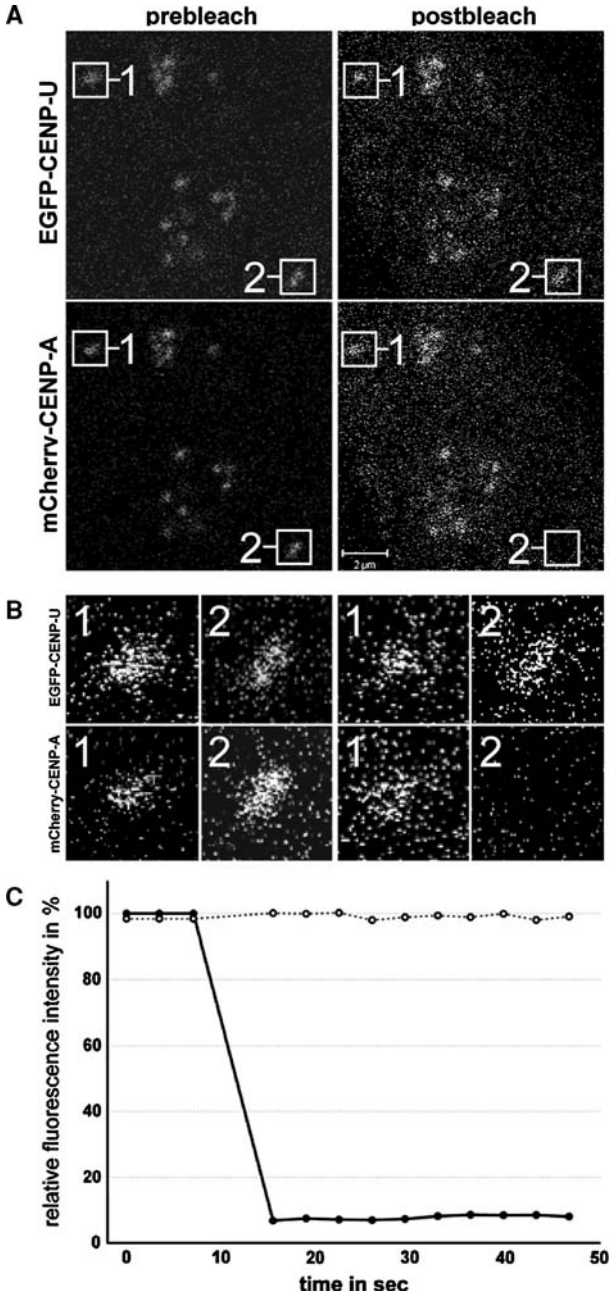


Fig. 1a–c Acceptor-bleaching FRET of the protein pair EGFP-CENP-U and mCherry-CENP-A. Human HEp-2 cells were co-transfected with both fusion proteins. A typical cell nucleus is displayed in **a** showing co-localisation at specific (centromeric) locations. Two of these locations, spots *1* and *2*, were selected for fluorescence intensity analysis before and after acceptor bleaching (see enlargement in **b**). Spot 1 served as control and showed no detectable intensity change. At spot 2, the acceptor fluorophore mCherry was bleached (compare pre-bleach and post-bleach in **a**, *lower two pictures*, and in **b**, *lower pictures*). In **c** the time course of the fluorescence intensity of the donor and the acceptor are shown. The acceptor intensities in the ROI were averaged and normalised to the mean intensity measured at the three time points before bleaching. The donor intensities in the ROI were averaged and normalised to the intensity measured at the first time point after bleaching. Bleaching of the acceptor (*closed circles*) did not result in a major fluorescence intensity change of the donor (*open circles*) indicating the absence of FRET

bleached at a centromeric region (spot 2 in Fig. 1a, lower left picture: before; lower right picture: after photobleaching; see also enlarged spots in Fig. 1b) while spot 1 served as control yielding the E_{var} values. Despite acceptor photobleaching, hardly any fluorescence intensity change in EGFP-CENP-U was observed (spot 2 in Fig. 1a upper left picture: before; upper right picture: after photobleaching; see also enlarged spots in Fig. 1b). The time course of the fluorescence intensity at spot 2 of the donor and the acceptor is shown in Fig. 1c; due to photobleaching, the acceptor fluorescence dropped to very low values of about 5% of the original and stayed low, while the donor fluorescence remained almost constant, unaffected by acceptor photobleaching. The E_{FRET} values (EGFP-CENP-U and mCherry-CENP-A in Fig. 2, grey bars) were distributed around 0% (14 kinetochores in 14 cells) and never exceeded the values of E_{var} (Fig. 2, black bars) which were measured in the same experiment at spot 1 where the acceptor was not photobleached. Thus, acceptor photobleaching had no measurable influence on the donor fluorescence intensity. EGFP-CENP-U showed no fluorescent energy transfer to mCherry-CENP-A, indicating that the N terminus of CENP-U is not in close proximity to the N terminus of CENP-A.

Correspondingly we analysed various other pairs of core kinetochore and NAC proteins including CENP-B. The 80-kDa CENP-B not only binds to the centromere but also to the pericentric heterochromatin domain distributed between sister kinetochores. It binds to a specific DNA sequence, the 17-bp CENP-B box, which is present in α-satellite repeat in human centromeres and in pericentromeric regions (Cooke et al. 1990; Ando et al. 2002). As for the co-expressed CENP-U and CENP-A, as well as for other protein pairs, the variation in donor fluorescence E_{var} at the non-bleached spot 1 was larger than the variation at the bleached spot 2, clearly indicating that no FRET occurred between the two tags at the N termini of these proteins (see Fig. 2, E_{var} black bars and E_{FRET} grey bars). For EGFP-CENP-T and mCherry-CENP-C, the variation distribution of the donor fluorescence intensities in the photobleached and the non-bleached spots overlaps well (16 kinetochores in as many cells). Also for the pairs EGFP-CENP-U with mCherry-CENP-C (13 kinetochores in 13 cells) and mCherry-CENP-M (14 kinetochores in 14 cells) as well as the pair EGFP-CENP-N with mCherry-CENP-C (12 kinetochores in 12 cells), photobleaching of the acceptor did not result in an increase in the donor fluorescence intensity, and the distributions coincided well. Among these five pairs, no FRET was observed.

The situation was different for the pair EGFP-CENP-U and mCherry-CENP-B. CENP-U colocalised with CENP-B (Fig. 3a). Again, two centromeric spots were selected (spot 1 and spot 2) and enlarged in Fig. 3b. The acceptor

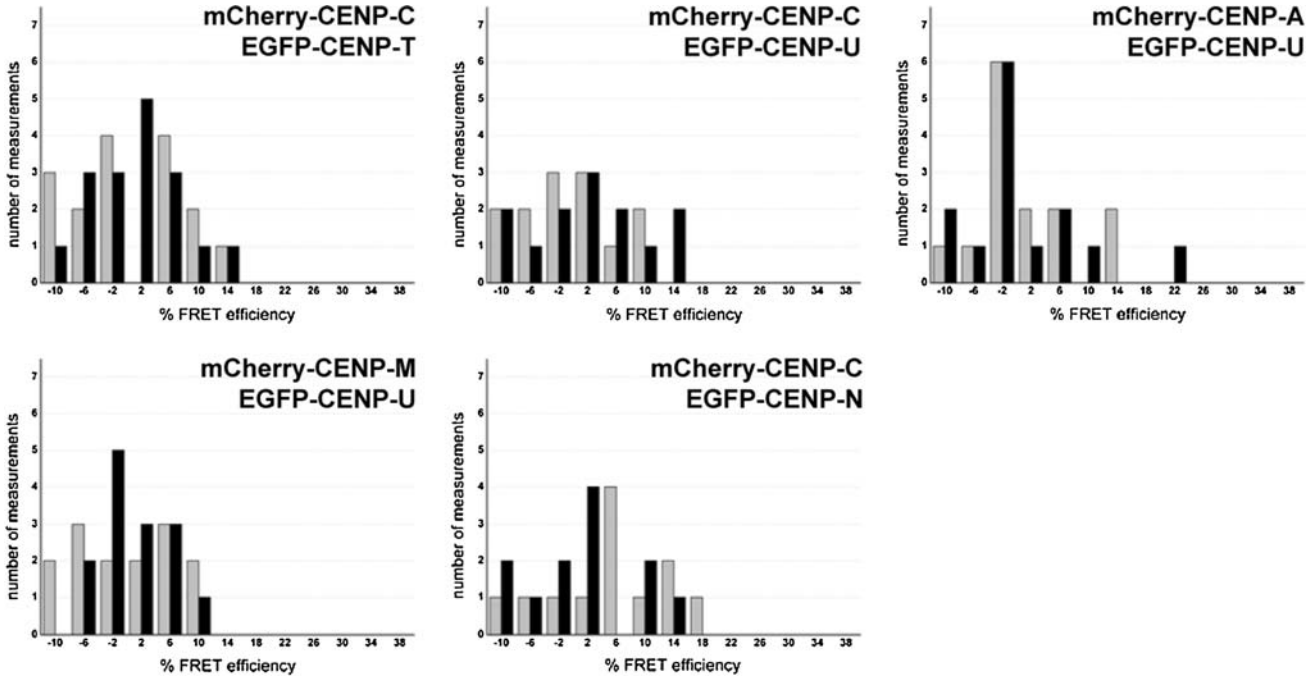


Fig. 2 Protein pairs not showing FRET. The donor fluorescence intensity variation observed during acceptor bleaching normalised to the intensity measured at the first time point after bleaching was determined for spot 1 (one example shown in Fig. 1) yielding E_{var} (*black bars*) and for spot 2 yielding E_{FRET} (*grey bars*). For the protein pairs indicated in the graphs, the number of observed single cases (grouped into E_{var} or E_{FRET} value ranges of 4%) is displayed versus the values of E_{var} or E_{FRET}. The value distributions of E_{var} and E_{FRET} coincide well, indicating the absence of FRET

mCherry was bleached at spot 2 (compare spot 2 pre-bleach and post-bleach in the bottom of Fig. 3a, b). Photobleaching had no detectable influence on the fluorescence intensity at spot 1 which served as control yielding the E_{var} values. The time course of the acceptor fluorescence intensity is plotted in Fig. 3c. Acceptor-photobleaching resulted in a nearly complete loss of fluorescence. During photobleaching, the donor fluorescence intensity increased (see Fig. 3c), indicating the presence of FRET. In 44% of the cases (18 kinetochores in 18 cells), the E_{FRET} value (Fig. 4, grey bars) was larger than the E_{var} value (Fig. 4, black bars). The max FRET efficiency values were relatively high (30%). Also for two other fusion protein pairs, Cerulean-CENP-M and EYFP-CENP-T (12 kinetochores in 12 cells) as well as Cerulean-CENP-I and EYFP-CENP-U (10 kinetochores in 10 cells), high values of E_{FRET} were observed (up to about 38%), and again, the E_{FRET} value was larger than the largest E_{var} value in about half of the cases (for Cerulean-CENP-I and EYFP-CENP-U as much as 70%), clearly indicating the presence of FRET. The single observations with $E_{FRET} > E_{var}$ however never reached 100% of all experiments; the remaining intensity changes in the donors of these pairs had values within the range of the variance E_{var} of the fluorescence intensities of the donor at the non-bleached spot. This indicates that not all but only about one-half or one-third of the protein pairs were observed in close vicinity and that quite a number of donors showed no FRET. Indeed, very high numbers of protein pairs showing FRET are not expected to be found since untagged endogenous proteins are also present in the cell, which mix with the tagged proteins so that in a number of cases the tagged donor will have an untagged protein as neighbour. This point will be explained in more detail in the section "Discussion". These data indicate that the N termini of these three protein pairs, CENP-I and CENP-U, CENP-M and CENP-T, and CENP-U and CENP-B, can be found in close proximity to one another.

In this study we used two different donor–acceptor pairs, EGFP with mCherry (Tramier et al. 2006) and Cerulean with EYFP (Orthaus et al. 2008). We studied one protein pair with both FRET pairs: we measured the proximity of CENP-M to itself by analysing EGFP-CENP-M and mCherry-CENP-M (15 kinetochores in as many cells) as well as Cerulean-CENP-M and EYFP-CENP-M (eight kinetochores). The obtained distributions of E_{FRET} and E_{var} were similar resulting in the same conclusion: in some but not many cases (about 23%) the N termini of two CENP-M proteins were measured to be close to one another (see Fig. 5a).

For a number of other protein pairs, the situation is ambiguous. In three cases, EGFP-CENP-N and mCherry-CENP-M (11 kinetochores), Cerulean-CENP-M and

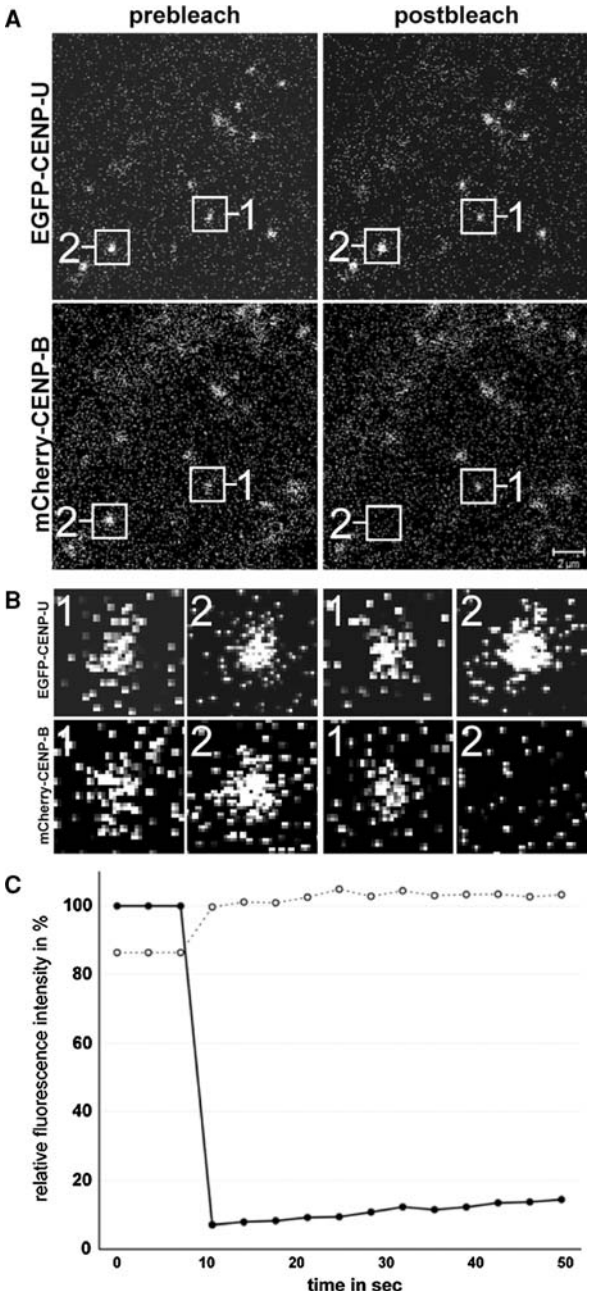


Fig. 3a–c Acceptor-bleaching FRET of the protein pair EGFP-CENP-U and mCherry-CENP-B. Human HEp-2 cells were co-transfected with both fusion proteins. A typical cell nucleus is displayed in **a** showing co-localisation at specific (centromeric) locations. Two of these locations, *spot 1* and *spot 2*, were selected for fluorescence intensity analysis before and after acceptor bleaching (see enlargement in **b**). Spot 1 served as control and showed no detectable intensity change. At spot 2, the acceptor fluorophore mCherry was bleached (compare pre-bleach and post-bleach in **a**, *lower two pictures*, and in **b**, *lower pictures*). In **c**, the time course of the fluorescence intensity of the donor and the acceptor is shown. The acceptor intensities in the ROI were averaged and normalised to the mean intensity measured at the three time points before bleaching. The donor intensities in the ROI were averaged and normalised to the intensity measured at the first time point after bleaching. Bleaching of the acceptor (*closed circles*) resulted in a fluorescence intensity increase in the donor (*open circles*) indicating the presence of FRET (with a FRET efficiency of about 14%)

EYFP-CENP-A (9 kinetochores) as well as Cerulean-CENP-T and EYFP-CENP-A (9 kinetochores), very high E_{FRET} values were measured, but only in a small number of single experiments (see Fig. 5b). Similarly, only in a small number of cases was E_{FRET} larger than the largest measured value of E_{var}. For other protein pairs: for mCherry-CENP-C and EGFP-CENP-M (13 kinetochores), mCherry-CENP-B and EGFP-CENP-M (11 kinetochores), mCherry-CENP-B and EGFP-CENP-T (14 kinetochores), mCherry-CENP-M and EGFP-CENP-M (15 kinetochores), EGFP-CENP-N and mCherry-CENP-B (18 kinetochores), EGFP-CENP-I and mCherry-CENP-M (18 kinetochores) as well as EGFP-CENP-I and mCherry-CENP-T (11 kinetochores), some but only a few E_{FRET} values were larger than the largest E_{var} value (Fig. 5a, b). In these cases, the few E_{FRET} values that were larger than E_{var} were not statistically significant. Thus in general, these protein pairs are not in close proximity to one another within the complex, and the few measured values of $E_{FRET} > E_{var}$ might be experimental errors. However, alternatively, during the cell cycle, complex structure or composition might alter so that in a few cases, the proteins are indeed close to one another allowing for FRET between the tags at their N termini. Here, the different interphase subphases G1, S-phase and G2 were not resolved (see the section "Discussion").

Discussion

At the centromere, the kinetochore proteins assemble including CENP-M, -N, -T, -U and -C. We showed here by acceptor-photobleaching FRET in living human HEp-2 interphase cells that in a high number of measured cases, the N termini of CENP-T and CENP-M, CENP-I and CENP-U, as well as CENP-B and CENP-U are in the direct neighbourhood of one another. Reduction of CENP-T eliminated CENP-M at centromeres (Foltz et al. 2006) consistent with our results. Surprisingly, yeast-two-hybrid experiments were not able to detect the association among any of these proteins studied (own unpublished data). On the other hand, for a number of proteins we did not observe single cases in which the N termini were close: CENP-U to CENP-M, -A and -C as well as CENP-C to CENP-T. Recently we showed that CENP-A is in close proximity to CENP-B at the centromeres in living human cells (Orthaus et al. 2008).

In the transfected cells, the fluorescently labelled proteins are present together with the corresponding unlabelled endogenous proteins. As a consequence, in the kinetochore complex the donor fusion proteins have acceptor-tagged as well as untagged proteins next to them. Our E_{FRET} value distributions therefore showed values of E_{FRET} similar to E_{var} for those donors without acceptors, and E_{FRET} values

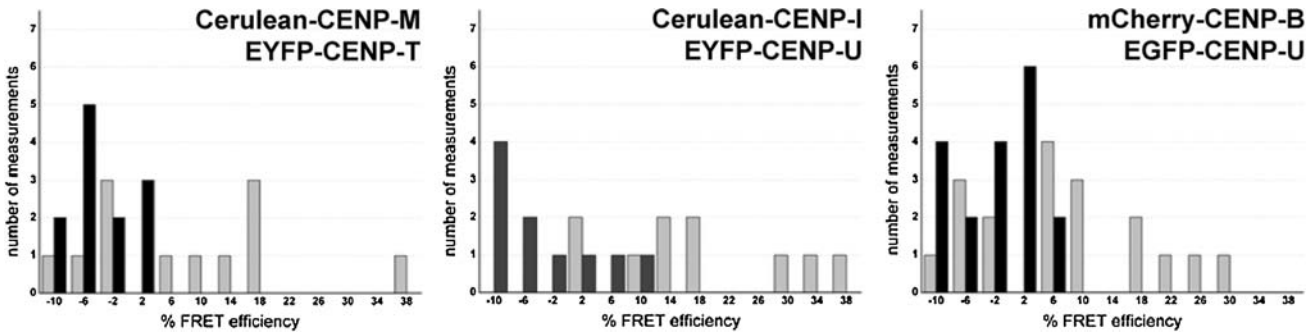


Fig. 4 Protein pairs showing FRET. The donor fluorescence intensity variation observed during acceptor bleaching normalised to the intensity measured at the first time point after bleaching was determined for spot 1 (one example shown in Fig. 3) yielding E_{var} (*black bars*) and at spot 2 yielding E_{FRET} (*grey bars*). For the protein pairs indicated in the graphs, the number of observed single cases (grouped into E_{var} or E_{FRET} value ranges of 4%) is displayed versus the values of E_{var} or E_{FRET}. Only some E_{FRET} values coincide with the distribution of E_{var} values; most E_{FRET} values exceed the E_{var} values, with E_{FRET} values of up to 38%, indicating the presence of FRET

larger than E_{var} for donors with acceptor molecules in close proximity. The number of donors in each situation is influenced by the expression levels of the tagged and the endogenous proteins, as well as by the level of incorporation of the tagged proteins into the kinetochore complex. Here we always selected cells with low expression levels of the tagged proteins, but that were high enough for high quality data, so that donor-only and acceptor-only complexes might be frequent. Observing donor molecules with high E_{FRET} values indicates that a complex was identified in which the donor fusion is in close proximity to the acceptor fusion. Our protein-exchange dynamics measurements of kinetochore proteins revealed that the inner kinetochore complex is not stable over the cell cycle but shows fast exchange of some proteins and stable incorporation of other proteins (Hemmerich et al. 2008); the kinetochore complex composition is thus flexible during the cell cycle. In our FRET results presented here, we did not distinguish different interphase cell-cycle phases. Therefore, donors with different E_{FRET} values might represent different complex compositions or complex structures differing with cell-cycle phase. We thus present the E values as measured and did not calculate mean values.

The FRET results for those protein pairs with only a few values of E_{FRET} larger than E_{var} (Fig. 5) can also be interpreted in terms of this view. These few cases with E_{FRET} larger than E_{var} are not necessarily due to experimental error but might indicate that these proteins are in direct proximity to one another only in less frequent or rare complex configurations, for example at selected time points during the cell cycle. Subphases during interphase were not resolved yet in our measurements for the following reasons: In order to distinguish G1 from S-phase and G2, we colour-labelled S-phase with PCNA fused to GFP or mRFP. Then, S-phase is identified by the particular cellular distribution of PCNA, G2 follows S-phase and G1 follows mitosis. This experimental approach works well for dynamic (e.g. FRAP) measurements when a single colour is sufficient for data acquisition (Hemmerich et al. 2008) since cells can be double-transfected with reasonable efficiency. For FRET experiments, the cells must be double-transfected to start with, thus an additional cell cycle marker (such as PCNA) would require a triple-transfection. Furthermore, the additional fluorophore might interfere with the FRET experiment. Nevertheless, cell-cycle-dependent FRET experiments are in the process of being established.

Here we observed by acceptor-bleaching FRET that in a few cases, the N terminus of CENP-T is in close proximity to that of CENP-A and CENP-B (see also Hellwig et al. 2008), as observed for several other protein pairs described here (Fig. 5). This interpretation suggests that CENP-T binds to the centromere at some distance to CENP-A but close enough that some CENP-T proteins can be detected in close vicinity to CENP-A. This interpretation fully agrees with the findings of Hori et al. (2008a) who applied different experimental approaches. Alternatively, CENP-T might bind to the centromere close to CENP-A only for a short period of time, while it binds far from CENP-A for a much longer time.

The data presented here as well as literature data are summarised in Table 1. FRET results are listed as (+) for close proximity between the two proteins and (−) when no FRET could be detected. Those cases with few positive FRET results only are listed with (?). When the acceptor-photobleaching FRET measurements were confirmed by lifetime FRET, the (+) and (−) symbols have a white centre. Literature data indicating a positive protein–protein interaction or proximity are listed with a circle while literature data indicating the absence of an interaction are represented by a square. Linker histone H1 was observed in the neighbourhood of CENP-A, -B and -C (Orthaus et al. 2009). The close vicinity of CENP-B to CENP-A and H1 was confirmed by bimolecular fluorescence complementation (BiFC; Orthaus et al. 2009). No FRET could be

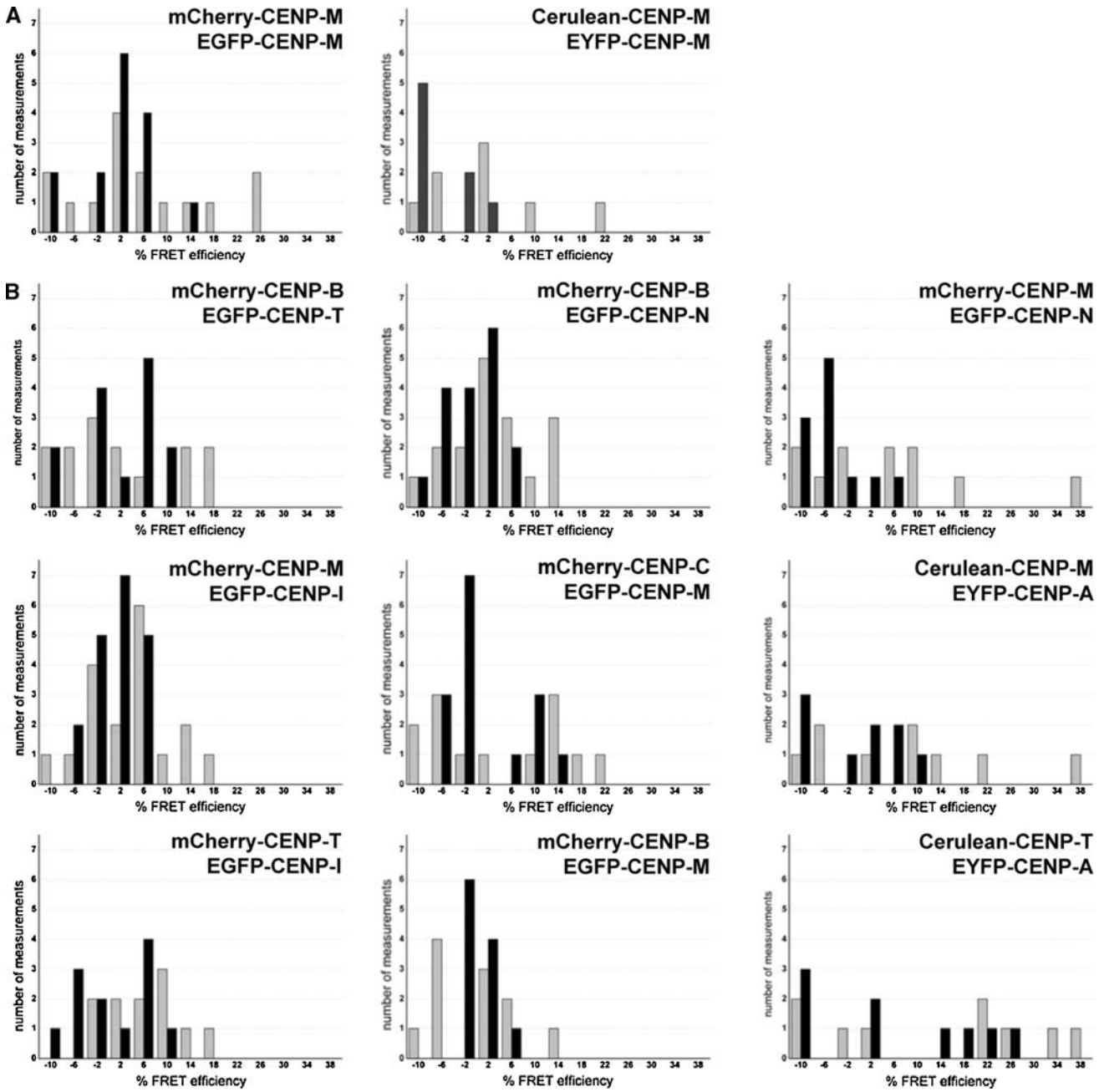
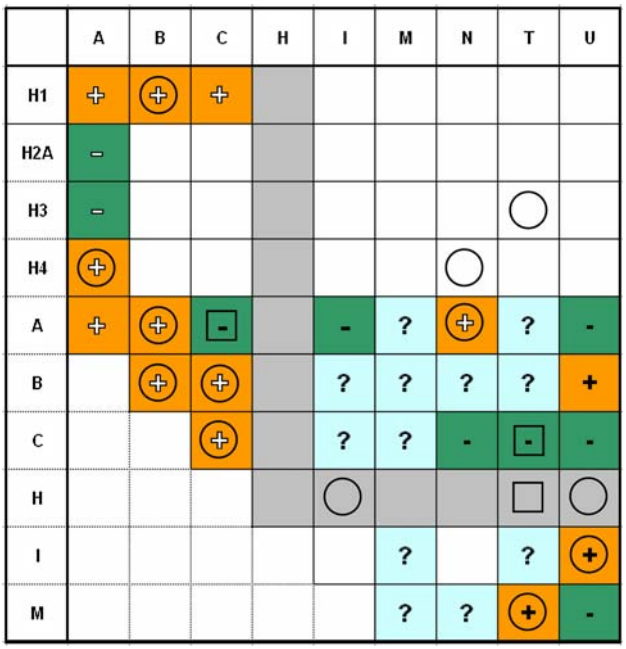


Fig. 5a, b Protein pairs rarely showing FRET. The donor fluorescence intensity variation observed during acceptor bleaching normalised to the intensity measured at the first time point after bleaching was determined for spot 1 yielding E_{var} (*black bars*) and for spot 2 yielding E_{FRET} (*grey bars*). For the protein pairs indicated in the graphs, the number of observed single cases (grouped into E_{var} or E_{FRET} value ranges of 4%) is displayed versus the values of E_{var} or E_{FRET}. Most E_{FRET} values coincide with the E_{var} values, while only some E_{FRET} values exceed them. **a** Comparison of the same protein pair using two different FRET pairs: EGFP and mCherry (*left*) or Cerulean and EYFP (*right*); **b** different protein pairs as indicated in the graphs

detected between CENP-A and the histones H2A and H3 all tagged at the N terminus (Orthaus et al. 2008). This is consistent with H2A, but not H3, being part of a CENP-A containing nucleosome. CENP-A was found to be in close vicinity to itself as well as to CENP-B (Orthaus et al. 2008) , to histone H4 (Black et al. 2007a, b; Black and Bassett 2008) and CENP-N (Carroll et al. 2009, unpublished) but not to CENP-C (Orthaus et al. 2008; Hori et al. 2008a), CENP-I (unpublished) and CENP-U (shown here). Recently, Carroll et al. (2009) showed that CENP-N is close to CENP-A and H4. We found CENP-B next to itself (unpublished; Pluta et al. 1992; Yoda et al. 1992; Kitagawa et al. 1995) and to CENP-C (unpublished; Suzuki et al. 2004) as well as CENP-C next to itself (Sugimoto et al.

Table 1 FRET results from this study as well as literature data



Our FRET results: *Plus signs* indicate close proximity between the two proteins. *Minus signs* indicate no FRET detected. *Question marks* indicate only a few positive FRET results. Acceptor-photobleaching FRET measurements confirmed by lifetime FRET are indicated by *symbols with white centres*. *Circles* represent literature data indicating a positive protein-protein interaction. *Squares* represent a measured absence of an interaction in the literature. CENP-H cells have a *grey background* since tagged CENP-H does not localise and was not used for FRET measurements (see "Results"). H2A might be placed next to CENP-A in centromeric nucleosomes, the N-termini however are too distal for FRET. In contrast, no FRET between H3 and CENP-A suggests that H3 is not (or hardly) present in centromeric nucleosomes

1997, unpublished). CENP-T is closely related to histone H3 but seems not to be associated with CENP-C and CENP-H (Hori et al. 2008a). Reduction of CENP-T eliminated CENP-M at centromeres potentially indicating these two kinetochore proteins being in close proximity to one another (Foltz et al. 2006). As members of the CENP-H–I complex, CENP-H and -I seem to be closely associated (Okada et al. 2006; Foltz et al. 2006; Nishihashi et al. 2002). CENP-U seems to interact with the CENP-H/CENP-I complex at the kinetochore (Minoshima et al. 2005). A yeast-two-hybrid experiment that detected interaction between CENP-M and CENP-H [mentioned as "unpublished" in Okada et al. (2006)] was not confirmed by our yeast-two-hybrid results (unpublished).

In conclusion, our in vivo measurements confirm in vitro results and yield additional data necessary to build an interaction network of core kinetochore and NAC protein components. Analysis of further protein pairs will progressively reveal the structural arrangements at centromeric chromatin.

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