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RNAi knockdown of human kinetochore protein CENP-H

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Abstract

The inner kinetochore protein complex binds to centromeres during the whole cell cycle. It serves as the basis for the binding of further kinetochore proteins during mitosis. CENP-H is one of the inner kinetochore proteins which is conserved amongst many eukaryotes. By specific RNAi knockdown, we reduced the CENP-H protein level in human HEp-2 cells down to less than 5% of its normal value. In these CENP-H knocked-down cells, we observed severe mitotic phenotypes like misaligned chromosomes and multipolar spindles, however, no mitotic arrest. Strong reduction of CENP-H resulted in a slightly reduced CENP-C level at the kinetochores and normal localisation of hBubR1, indicating a functional mitotic checkpoint at the hBubR1 protein level. In CENP-H knocked-down human cells, the misaligned chromosomes contained only reduced levels of CENP-E. Our data clearly indicate that CENP-H has an important impact on the architecture and function of the human kinetochore complex.

Keywords: Mitosis; Human kinetochore; CENP-H; CENP-C; hBubR1; CENP-E; Mitotic checkpoint; RNAi knockdown

At each mitosis, accurate segregation of every chromosome is ensured by the assembly of a kinetochore at each centromere locus (for reviews, see [1–6]). Chromosome segregation errors cause aneuploidy which can cause cancer (for reviews, see [7,8]). Traditional electron microscopy of chromosomes revealed that the kinetochore of vertebrate cells is a trilaminar structure on the surface of the centromeric chromatin [3]. The inner kinetochore plate has an essential role in kinetochore assembly, and the outer kinetochore plate is both, a mitotic checkpoint and a microtubule binding structure. In vertebrates, several proteins are supposed to build the inner kinetochore complex which localise to a core domain of centromeric chromatin and which are present at the centromere during the whole cell cycle. Among these proteins are CENP-A, CENP-B CENP-C, CENP-H, CENP-I, and Mis12 [4]. With the exception of CENP-B, these foundation kinetochore proteins are essential: they are found at all active (but not at inactive) centromeres including neocentromeres. Their depletion results in chromosome missegregation and disruption of mitosis.

Central to the kinetochore protein assembly is CENP-A which replaces histone H3 at the centromeric nucleosome. This protein is present in all eukaryotes and its depletion leads to the mislocation of kinetochore proteins like CENP-B and CENP-C [9–11]. CENP-A nucleosomes directly recruit a CENP-A nucleosome proximal-associated complex (NAC) comprised of the three centromere proteins CENP-M, CENP-N, and CENP-T, along with CENP-U(50), CENP-C, and CENP-H. Assembly of the CENP-A NAC at centromeres is dependent on CENP-M, CENP-N, and CENP-T. Seven CENP-A-nucleosome distal (CAD) centromere components (CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-R, and CENP-S) are found to assemble on the CENP-A NAC. The CENP-A NAC is essential, as disruption of the complex causes errors of chromosome alignment and segregation that preclude cell survival despite continued centromere-derived mitotic checkpoint signalling [12,13]. The loading of CENP-A onto the centromeric DNA appears to be one of the early steps that determine the centromere identity, although mistargeting of CENP-A to

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DNA outside the conventional centromere regions is not sufficient per se to induce the formation of functional neocentromeres, while it seems to re-direct CENP-C to these sites [10,14]. This suggests that other factors including particular DNA sequences or structures may be required for this process [15]. CENP-B is the only inner kinetochore protein to bind to a specific centromere sequence, the 17 bp CENP-B box, which occurs in a subset of α-satellite repeats in humans [16–20]. CENP-B is non-essential: it is absent from functional centromeres that lack CENP-B boxes and CENP-B-null mice show normal viability and correct localisation of other kinetochore proteins [21]. CENP-C binds centromeric DNA of the same type of α-satellite DNA as CENP-B [15,22,23] but without sequence specificity [24]. The interaction between CENP-B and -C has been shown by yeast-two-hybrid and *in vitro* interaction assays [25], nevertheless the exact arrangement of these two proteins remains unresolved. CENP-C kinetochore localisation depends on CENP-A in mouse cells [10] and, in addition, on CENP-H and CENP-I in chicken [26,27]. Like CENP-A, CENP-C is found only at active centromeres [28] and is needed to form a functional kinetochore. In chicken, the absence of CENP-C resulted in mitotic arrest, chromosome misaggregation, and apoptosis [26,29].

With the kinetochore proteins CENP-A and CENP-C, also CENP-H localises constitutively to the inner kinetochore in both interphase and metaphase in mouse fibroblast [30] and human HeLa cells [31]. CENP-H is detected at neocentromeres but not at inactive centromeres in stable dicentric chromosomes. *In vitro* binding assays suggest that CENP-H binds to itself and mitotic centromere-associated kinesin (MCAK) [31], but not to CENP-A, -B, or -C. In addition, in *in vitro* binding studies, an interaction of CENP-H with the N-terminus of CENP-I (unpublished, from [27]) and with Hec1 (a member of the Ndc80 complex [32]) was found. Immuno-cytochemical analysis of the CENP-H-deficient chicken DT40 cells demonstrated that CENP-H is necessary for CENP-C but not CENP-A localisation to the centromere [31]. Analysis of a conditional knockout of CENP-H in DT40 cells revealed that CENP-H is essential for cell growth and mitotic progression [31]. In chicken DT40 cells, a CENP-H derivative that lacks the amino-terminal 72 amino acids (aa) and the carboxy-terminal 10 aa, complemented CENP-H function [32].

Also CENP-I is a constitutive centromere protein that localises to the centromere throughout the cell cycle [27,33,34]. The chicken DT40 phenotype of CENP-I knockout cells is similar to that of CENP-H knockout cells. Analyses of both CENP-H and CENP-I knockout chicken cells suggested that CENP-H and CENP-I are mutually interdependent for targeting to the pre-kinetochore structure and that both are necessary for CENP-C localisation to centromeres [26,27]. The CENP-H-I complex, which includes the established inner kinetochore components CENP-H and CENP-I, and additionally seven other proteins (CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-U(50), and CENP-M), is required for the efficient incorporation of newly synthesised CENP-A into centromeres in human and chicken [13].

Mitotic checkpoint proteins bind to the inner kinetochore complex during mitosis and are activated at the kinetochores of unattached chromosomes. These activated checkpoint components subsequently inhibit the anaphase-promoting complex and prevent the ubiquitination of substrates whose destruction is required for advance to anaphase (reviewed by [3]). A single unaligned chromosome is sufficient to inhibit anaphase onset, correlating with the presence of an activated checkpoint at the kinetochore [35,36]. CENP-E is a kinesin-like motor protein localised at the outer kinetochore. It is required for efficient capture and attachment of spindle microtubules by kinetochores, a necessary step in chromosome alignment during prometaphase [37]. Functional disruption of CENP-E by various methods consistently resulted in the appearance of some unaligned chromosomes at metaphase [38]. CENP-E interacts with the checkpoint protein BubR1 [38,39] and stimulates directly its kinase activity [40,41]. This leads to a Mad2 dependent mitotic arrest. Kinetochores without CENP-E cannot activate the BubR1 kinase [42]. Without CENP-E, checkpoint function cannot be established or maintained *in vitro* [43] or in mice [37]; it is required for prevention of premature advance to anaphase in the presence of unattached kinetochores [37]. BubR1 kinase activity is silenced after spindle assembly and chromosome alignment.

The inner kinetochore proteins preferentially form at α-satellite repetitive DNA sequences although the presence of this DNA does not define kinetochore complex settlement. Furthermore, kinetochore formation is initiated by the loading of CENP-A which however, at least *in vitro*, does not show interactions with other kinetochore proteins. Thus, centromeric chromatin structure might play a role in kinetochore function. A detailed analysis resolved the function of CENP-H and elucidated the protein binding hierarchy of the inner kinetochore complex in chicken DT40 cells [32,44]. Here, we analysed CENP-H RNAi knockdown in human HEp-2 cells in order to determine the influence of CENP-H on kinetochore function and the binding hierarchy in human kinetochores.

Materials and methods

Cell culture of HEp-2 cells. HEp-2 (HeLa derivative) cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, USA) and grown to subconfluency as recommended. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) in a 9.5% CO_2 atmosphere at 37 °C. After aspiration of the medium, cells were washed with magnesium- and calcium-containing PBS (Sigma–Aldrich, Taufkirchen, Germany) followed by detachment with trypsine/EDTA (PAA Laboratories, Pasching, Austria), centrifugation for 2 min at 2000 rpm, and re-seeding, for instance on coverslips.

Antibodies and immuno-fluorescence. The following primary antibodies were used for indirect immuno-fluorescence analyses: human CREST sera

against centromere proteins CENP-A, CENP-B, and CENP-C [45], mouse monoclonal anti-β-tubulin antibody (Sigma–Aldrich, Taufkirchen, Germany), mouse monoclonal antibody against CENP-A (MBL, Woburn, USA), guinea pig serum against the N-terminal half of CENP-C (a kind gift of K. Yoda), and rabbit polyclonal antibody against hBubR1 and rabbit polyclonal antibody against CENP-E (kind gifts of T.J. Yen). Cells grown on coverslips were fixed by incubation in 4% paraformaldehyde for 10 min at room temperature followed by 5 min permeabilisation in 0.25% Triton X-100 (Serva, Heidelberg, Germany). Immuno-fluorescence was performed as described previously [46]. For immuno-fluorescence staining, primary antibodies from mouse, rabbit, guinea pig, and human sources were used and detected with species-specific secondary antibodies linked to FITC or rhodamine (Jackson Immunoresearch, West Grove, USA). Cellular DNA was stained with ToPro3 and DAPI (Molecular Probes, Eugene, USA) at concentrations established individually. The coverslips were then mounted onto microscope slides using Vectashield Mounting Medium (Vector Lab, Burlingame, USA). Microscopic images were collected by using an Axiovert 200M/LSM510META microscope (Carl Zeiss, Jena, Germany). Samples were scanned using a 63×/1.40 Plan-Apochromat oil objective. GFP/FITC, rhodamine, and ToPro3 dyes were excited by laser light at 488, 543 or 633 nm wavelength, respectively. To avoid bleed-through effects in double or triple staining experiments, each dye was scanned independently (multitrack mode). Fluorescence signals were detected using narrow band pass (±20 nm wavelengths) instead of long pass filters. Thus, only the peak regions of the fluorescence signals were taken for data analysis. Single optical sections were selected either by eye-scanning the sample in z axis for optimal fluorescence signals, or taken from stack projections. Images were electronically merged and stored as TIF files. Figures were assembled from the TIF files using Adobe Photoshop software. The fluorescence intensity analysis at centromeres was performed using Metamorph Offline 6.1r4 software (Universal Imaging Corporation, Downingtown, PA).

Immuno-blotting. HEp-2 cells were harvested as described above, stained with Trypan blue 0,4% (Eurobio, Courtaboeuf Cedex B, France), counted in a Neubauer chamber to determine the cell number, and lysed for 10 min at 100 °C in an appropriate volume of 2% SDS, 0.1% bromophenol blue, 35 mM dithiothreitol, 25% glycerin, and 60 mM Tris–HCl, pH 6.8. Cell lysates with equal amounts of total protein were separated by SDS–PAGE and blotted onto Protran BA nitrocellulose (Schleicher & Schuell, Dassel, Germany). Proteins reacted with human anti-splicing factor SmB/B′ antibody (WM Keck Autoimmune Disease Center, La Jolla, USA), mouse monoclonal antibody against lamin A/C (#sc-7292, Santa Cruz Biotechnology, Santa Cruz, USA), goat anti-CENP-H polyclonal antibody (#sc-11297, Santa Cruz Biotechnology, Santa Cruz, USA), guinea pig serum against the N-terminal half of CENP-C (a kind gift of K. Yoda), rabbit polyclonal antibody against hBubR1, and rabbit polyclonal antibody against CENP-E (kind gifts of T.J. Yen). Bound antibodies were detected with horse radish peroxidase-conjugated goat anti-human (#109-035-097), goat anti-mouse (#115-035-072), goat anti-guinea pig (#106 035 006), and goat anti-rabbit (#111 035 006) IgG antibodies (Jackson Immunoresearch Laboratories, West Grove, USA) at a dilution of 1:4000 and mouse anti-goat IgG antibody (#sc-2354, Santa Cruz Biotechnology, Santa Cruz, USA) at a dilution of 1:250, 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). The protein amount in the corresponding bands was analysed using the Phoretix TotalLab software (Biostep, Jahnsdorf, Germany).

RNA interference. 5′-UGGUUGAUGCAAGUGAAGA-3′ (top strand) siRNA was synthesised (Qiagen–Xeragon, Germantown, USA) for RNAi against CENP-H specific for the N-terminal portion of CENP-H. As a positive control for protein synthesis knockdown, the RNA sequence for lamin A/C (5′-CUGGACUUCCAGAAGAACAdTdT-3′) was used. The procedure for RNAi was adopted from published protocols [47]. HEp-2 cells were grown to about 50% confluence in coverslip-containing six-well plates. Cells were transfected with 5 μg of the double-strand siRNA using RNAiFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Repeatedly, after 24 h time steps the cells were harvested and cell viability properties were monitored using Trypan blue. In addition, coverslips were stained with antibodies to monitor the RNAi induced phenotypes and the presence of further kinetochore proteins. Aliquots of the cells were lysed (see above) and analysed by Western blotting in order to verify the protein knockdown of the RNAi treatment and the amount of further kinetochore proteins. For cell cycle examination FACS analysis was performed with another aliquot of the cells. In order to determine the siRNA transfection efficiency, a CY3 labelled Luciferase GL2 RNA duplex (CY3-5′-CGU ACG CGG AAU ACU UCG A dTdT-3′; Dharmacon, Lafayette, USA) was applied and transfected cells were counted.

Analysis of the CENP-H genotype of HEp-2 cells. In the siRNA binding domain, the CENP-H gene contains a polymorphism which was detected by reverse transcriptase (RT)-PCR of HEp-2 cells. RNA was isolated from HEp-2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First strand cDNA was derived from oligo(dT) primed reverse transcription by the use of Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions.

For determination of the respective genotype in HEp-2 cells, about 20 ng of genomic DNA was used to PCR amplify the different CENP-H isoforms using Ready-To-Go PCR beads (Amersham). Seminested PCR conditions were one cycle of denaturation at 93 °C for 60 s, followed by 5 cycles of denaturing at 95 °C for 60 s, annealing at 58 °C for 30 s, and extension at 72 °C for 90 s; followed by 30 cycles of denaturing at 95 °C for 60 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s; and 1 cycle of final extension at 72 °C for 5 min. PCR products were purified by precipitation and sequenced with the same primers used for PCR amplification by the dye terminator method using BigDye v3.1 (Applied Biosystems).

For amplification of genomic DNA and subsequent sequencing of the resulting amplicons that correspond to the CENP-H isoforms listed in Fig. 1, we used for full-length isoform BG 742599 the primers BG/BFa-for (5′-cgt ttg cct gtt gag tgg ta-3′) and BGa-rev (5′-ggt gga cag aca aat gca ca-3′) in the first PCR, and BG/BFa-for and BGi-rev (5′-caa ttt cct taa ggg cag ga-3′) in the second PCR; for BF 245236 the primers BG/BFa-for and BFa-rev (5′-tct cca tct gta ggt ttt gtc g-3′) in the first PCR, and

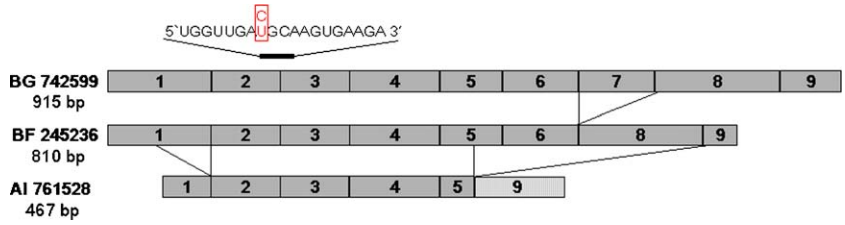

Fig. 1. Splice variants of the CENP-H gene and position of the siRNA target sequence within the area of exon 2 and 3. The clones BG742599 (915 bp), BF245236 (810 bp), and AI761528 (467 bp), which represent the 3 splice variants, were obtained from RZPD (Berlin, Germany). Their names correspond to their accession number in PubMed. The lanes show which areas are removed by splicing in the different forms. Human HEp-2 cells only contain the BF245236 splice variant. In HEp-2 cells, a single nucleotide polymorphism (SNP) from T to C (boxed in) was found in the area of siRNA (marked with a thick lane).

BG/BFa-for and BFi-rev (5′-tgt cca aat caa tct tct gtt tg-3′) in the second PCR; for AI 761528 the primers AIa-for (5′-cag gct gag agc aca gac aa-3′) and AIa-rev (5′-tga aca ctg ctt cat ccg ag-3′) in the first PCR, and AIa-for and AIi-rev (5′-gga acc cat tcc ctc aaa ct-3′) in the second PCR. In case of BF 245236 the amplicons obtained by RT- and seminested PCR were cloned into PCR2.1-TOPO (Invitrogen, Carlsbad, USA) and propagated in *Escherichia coli* TOP10 cells. Plasmids were isolated from several isolated clones and their inserts sequenced using plasmid primers.

Cell viability assays. DAPI staining of DNA was used to assess the 'loss of viability' phenotypes in CENP-H depleted cells. Mitotic indices were determined by ToPro3 and centromere staining, and quantification of mitotic cells in confocal images ($n > 400$). In parallel, HEp-2 cells were analysed for frequency of living cells. The supernatant as well as the trypsine/EDTA-detached cells were combined, washed twice in phosphate-buffered saline (PBS), and pelleted at 2000 rpm. The pellet was redissolved in 1 ml PBS. Fifty microliters of this cell suspension was mixed with 50 µl Trypan blue 0,4% (Eurobio, Courtaboeuf Cedex B, France) and immediately loaded onto a Neubauer chamber. Trypan blue exclusion served to discriminate and quantitate dead and living cells.

Cell cycle analysis and cell synchronisation. Cells were harvested and fixed with 95% EtOH for 15 min at 4 °C and stained with 20 µg/ml propidium iodide (PI, Sigma–Aldrich, Taufkirchen, Germany) in phosphate-buffered saline (PBS) with 250 µg RNAse and 1% FCS for 30 min at 37 °C. Between incubations, cells were washed with PBS containing 1% FCS. Subsequent flow cytometry was performed with an FACScan cytometer (Becton–Dickinson, Franklin Lakes, USA). Fluorescence data were displayed as histogram blots using Cell Quest analysis software (Becton–Dickinson, Franklin Lakes, USA). As a control for correct cell cycle analysis, we synchronised HEp-2 cells at different stages of the cell cycle. Incubation of HEp-2 cells with serum-free medium for 24 h yielded in an enrichment of cells in G_0 phase. For a mitotic arrest, we incubated freshly seeded HEp-2 cells with 1 µg/ml nocodazole (Sigma–Aldrich, Taufkirchen, Germany) for 12 h. For enrichment of cells in S-phase, we applied 1 µg/ml aphidicoline (Sigma–Aldrich, Taufkirchen, Germany) for 12 h followed by subsequent PI-staining and FACS analysis or we performed a double thymidine block. Therefore, HEp-2 cells were grown to 50% confluence and incubated with 5 mM thymidine (Sigma–Aldrich, Taufkirchen, Germany) for 16 h. Cells were washed with PBS and cultivated for 10 h with DMEM + 10% FCS. After repeated thymidine treatment for 16 h, cells were subsequently harvested, stained, and analysed in the cytometer.

Results

CENP-H localises constitutively to the inner kinetochore plate and plays a fundamental role in organisation and function of the active human centromere/kinetochore complex [31]. In chicken DT40 cells, CENP-H localises to the kinetochore during the whole cell cycle and was found to be necessary for CENP-C, but not CENP-A, localisation to the centromeres [44]. In the absence of CENP-H, these chicken cells were arrested in metaphase consistent with loss of centromere function [44]. Many knockout/down experiments in different organisms elucidated protein functions and interdependencies in kinetochore assembly. We were interested in essential functions of CENP-H in human cells. We therefore studied CENP-H RNAi knockdown in human HEp-2 cells.

The siRNA leads to depletion of CENP-H in human HEp-2 cells

In different tissues, CENP-H appears in several isoforms (see harvester.embl.de/harvester/Q9H3/Q9H3R5.htm; Fig. 1). We designed a suitable siRNA sequence which simultaneously knocked-down all CENP-H isoforms but no other protein (verified by BLAST analysis). The target sequence (dTGGTTGATGCAAGTGAAGA) is situated at the exon 2 to 3 transition (see Fig. 1) starting at gene position 177. Then, we determined which of the three available isoforms (Fig. 1) occurred in HEp-2 cells. HEp-2 cell RNA was isolated and RT-PCR was performed in order to obtain the HEp-2 cDNA. Semi-nested PCR with a specific primer pair for each isoform (see Materials and methods) leads to the identification of BF245236 as the CENP-H form present in HEp-2 cells: a PCR fragment of 810 bp length was identified in a 1% agarose gel (data not shown). Subsequently, the PCR fragment was cloned and several clones were sequenced. Sequence comparison of several (96) clones identified a single nucleotide polymorphism (SNP) at sequence position 8 of the siRNA from T to C (boxed in, Fig. 1). Nevertheless, the further analyses indicated that the selected and synthesised siRNA indeed knocked-down CENP-H specifically (see Fig. 2). For the following set of experiments including cell number determination, Western blot analysis, immuno-fluorescence, and flow cytometry analysis, equal numbers of HEp-2 cells were seeded in six-well plates, transfected with siRNA, and incubated for 24, 48, or 72 h. At these different time points, the cells were subjected to the various experimental analyses. Using Cy3-labelled luciferase GL2 RNA duplex, we measured the siRNA transfection efficiency and found it to be close to 100% (data not shown). After 24, 48, and 72 h incubation with siRNA, cells were harvested, counted, lysed, and equal numbers of cells were analysed by Western blots. Fig. 2A displays the very low protein level of CENP-H after 72 h of incubation in comparison to untreated cells. As a positive control, also lamin A/C was knocked-down by lamin-specific siRNA in HEp-2 cells (Fig. 2B, compare [48]). To control the specificity of the siRNA against CENP-H and to ensure equal amounts of other proteins in control and siRNA treated samples, we stained the cell lysates with an antibody against the splicing factor SmB/B′ and found similar protein levels in control and CENP-H depleted cells (Fig. 2C). Western blots were quantitatively analysed after incubation times of 24, 48, and 72 h. After 72 h, the protein level of CENP-H was reduced to a few percent (<5%) compared to the initial value (data not shown).

Depletion of CENP-H in human cells resulted in aberrant mitotic phenotypes and decreased numbers of living cells but did not lead to mitotic arrest

In the course of cytological analysis of CENP-H depleted human cells, we performed an indirect immuno-fluorescence analysis. HEp-2 cells grown on coverslips for 24, 48, and 72 h were fixed, permeabilised, stained with ToPro3 (DNA), CREST (human anti-centromere serum against CENP-A, CENP-B, and CENP-C), and anti-β-tubulin (binding spindle microtubules), and analysed in a Zeiss

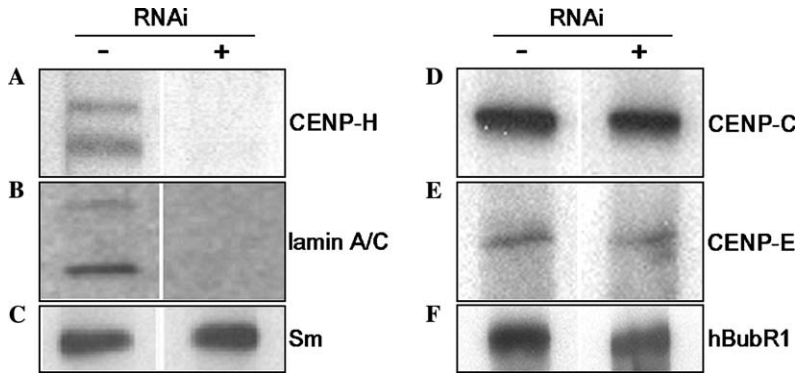

Fig. 2. CENP-H reduction by RNAi did not influence the cellular content of the kinetochore proteins CENP-C, CENP-E, and hBubR1. RNAi was used to block the protein synthesis of CENP-H (A, C–F) and, as a positive control, lamin A/C (B) in HEp-2 cells. The reduction of protein level and the amount of further proteins were then analysed by immuno-blotting of protein lysates from cells treated without (−) or with (+) RNAi oligonucleotides for 72 h. (A) The amount of CENP-H was strongly reduced by RNAi. In Western blots using a goat anti-CENP-H antibody, CENP-H always appeared as a double band. The lower band of the two bands migrated at 30 kDa, the expected size of CENP-H. The upper band appeared at about 32 kDa. (B) SiRNA against lamin A/C leads to the depletion of both protein isoforms. (C) An anti-splicing factor SmB/B′ antibody was used as a loading control in untreated and CENP-H depleted cells (Sm). Depletion of CENP-H did not affect the protein amount of CENP-C (D), CENP-E (E) or the checkpoint protein hBubR1 (F). The protein content did not change in control and siRNA treated cells.

CLSM 510Meta microscope (see Fig. 3a). The CENP-H down-regulated HEp-2 cells showed major aberrant mitotic phenotypes: In most mitotic cells, we observed a variable number of misaligned chromosomes (Fig. 3a-A); a considerable amount of chromosomes were not correctly aligned at the metaphase plate in a bipolar manner (metaphase plate indicated by arrows in Fig. 3a-A). In some mitotic cells, we found multipolar spindles (more than two spindle poles; see arrowheads in Fig. 3a-B). In only a few mitotic cells, we observed strongly condensed chromosomes (Fig. 3a-C) indicating the apoptotic state. The strongly condensed chromosomes could be identified by their rounded shape. They were spread over the entire spindle microtubule apparatus. These phenotypes were also observed in chicken DT40 cells after CENP-H depletion [44].

To analyse the effect of these aberrant mitotic forms on the cell proliferation, we measured the number of surviving human HEp-2 cells with down-regulated CENP-H. In order to discriminate between living and dead cells, siRNA treated and control cells were stained by Trypan blue (staining dead cells) and counted in a Neubauer chamber. We observed that the number of surviving siRNA treated cells was lower compared to controls with normal levels of CENP-H (Fig. 4a). After 72 h, the number of living CENP-H deficient cells was about half (52%) compared to control cells (see Fig. 4a). This result indicates an inhibition of proliferation or increased cell death due to CENP-H depletion.

Then, we determined the number of aberrant mitotic cells in order to quantify the aberrant mitotic phenotypes having multipolar spindles or misaligned chromosomes (see Fig. 4b). Four hundred and fifty cells per time point were counted in six independent experiments. The number of cells is given relative to the number of all cells observed. With increasing incubation time, control cells showed a slightly decreasing number of aberrant mitotic cells (18% after 24 h, 15% after 48 h, and 11% after 72 h; see Fig. 4b). Of all cells observed, only 1% showed multipolar spindles after 48 h; most of the aberrant control cells displayed the misaligned chromosome phenotype which decreased from 18% to 13% and 10% after 24, 48, and 72 h, respectively (Fig. 4b). This situation was different in the siRNA treated cells: here the number of aberrant mitotic cells was 1.7× higher compared to control cells after 24 h, 2.5× higher after 48 h, and 4.3× higher after 72 h. CENP-H depleted cells established multipolar spindles with a frequency of 2% after 24 h, 1% after 48 h, and 4% after 72 h. In addition, like in the control, we observed a high number of mitotic cells with misaligned chromosomes increasing from 30% (24 h) up to 35% (48 h), and 43% (72 h). The appearance of this phenotype increased with time (while it decreased for the control cells) and it was of considerably higher number. We thus conclude that in human HEp-2 cells, depletion of CENP-H results in a strongly increased number of abnormal mitotic phenotypes.

The high and increasing number of observed abnormal phenotypes of siRNA knocked-down HEp-2 cells indicates that CENP-H is highly important for normal cell cycle progression and chromosome segregation. We therefore tested if CENP-H depletion resulted in mitotic arrest in human cells as it did in chicken. We thus performed a FACS analysis using propidium iodide for DNA staining in order to count cells with either di—(2N) or tetraploid (4N) sets of chromosomes—or even more in case of severe distortions. As controls, cells were blocked in G_0 phase by serum starvation, in S-phase by adding aphidicoline or thymidine, or in mitosis by adding nocodazole. We observed the expected enrichment of cells in the respective cell cycle phase (data not shown). The HEp-2 cells were analysed in time steps of 24 h from day 1 to day 9 (216 h; see Fig. 5). We observed a normal cell cycle distribution although the cells were depleted of CENP-H. Thus, the cell cycle of CENP-H depleted HEp-2 cells seemed to be normal without

indications of cell cycle delay and distorted chromosome segregation. This finding was supported by observations in chicken, where the spindle checkpoint became inactive after 72 h in CENP-H$^{-/-}$ DT40 cells most likely reflecting complete removal of CENP-H. However, in contrast to the situation in human HEp-2 cells, Fukagawa et al. [44] reported distorted chromosome segregation and a metaphase arrest 48 h after knocking out CENP-H in chicken. The abnormal mitotic phenotypes resulting from depletion of CENP-H in human cells either were corrected in a short time or the affected cells died.

CENP-H depleted kinetochores showed an unchanged presence of the checkpoint protein hBubR1 and a reduced presence of CENP-C and CENP-E

In order to elucidate the architecture and functionality of the kinetochore complex, we studied the localisation of several kinetochore proteins in CENP-H depleted HEp-2 cells. In a first step, we wanted to get insight into the interdependencies between CENP-H and CENP-C in human cells since CENP-H is necessary for the recruitment of CENP-C to kinetochores in chicken DT40 cells [44]. We therefore stained HEp-2 cells with antibodies against CENP-A and CENP-C, and compared the distribution of these kinetochore proteins in control and siRNA treated mitotic cells (150 mitotic cells in 3 different experiments, Fig. 3b). CENP-A was used as a marker for the kinetochore. CENP-C is a constitutive kinetochore protein stably binding to the complex. In healthy cells, CENP-A and CENP-C colocalised at the kinetochore throughout the whole cell cycle (Fig. 3b-A). In CENP-H depleted human cells and contrast to chicken DT40 cells, we could detect considerable amounts of CENP-C, even at kinetochores of misaligned chromosomes (arrowhead in Fig. 3b-B). In addition, we observed

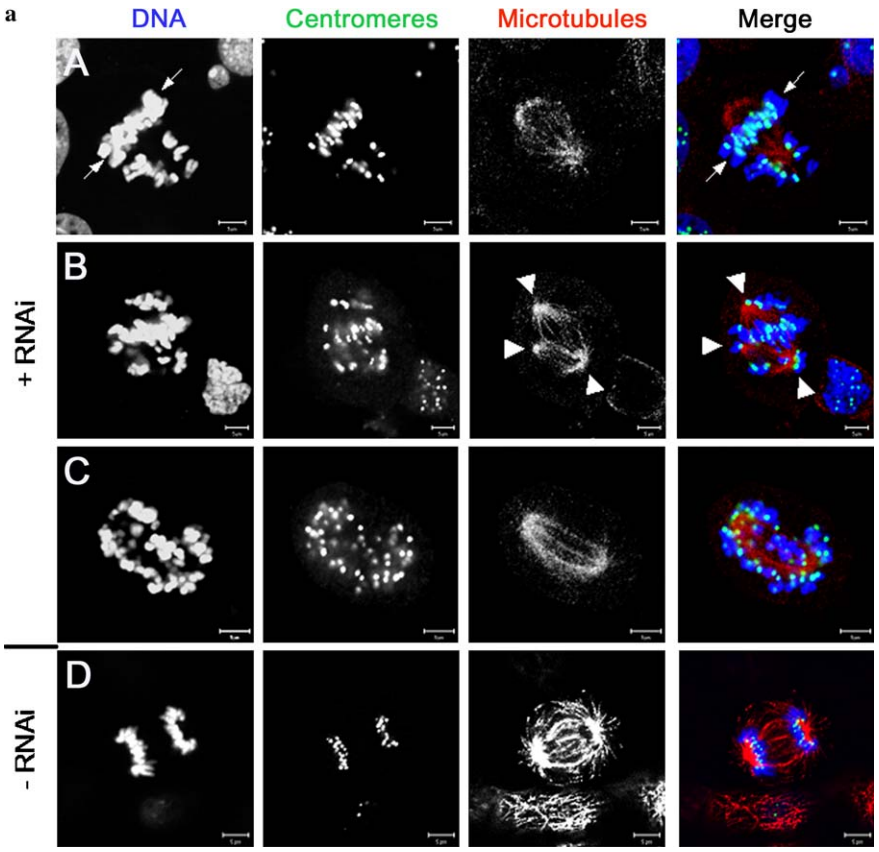

Fig. 3. (a) Depletion of CENP-H leads to aberrant mitotic phenotypes. CENP-H deficient cells displayed aberrant chromosome alignment and multipolar spindles, which possibly lead to apoptosis. The mitotic phenotypes of cells treated with RNAi against CENP-H were assessed by triple fluorescence staining of spindle microtubules (β-tubulin, red), centromeres (ACA, green), and DNA (ToPro3, blue). (A) Metaphase cell with misaligned chromosomes. The position of the metaphase plate is indicated by arrows. (B) Mitotic cell with a multipolar spindle. The 3 spindle poles are marked with arrowheads. (C) Mitotic cell with strongly condensed chromosomes indicated by their rounded shape. (D) Untreated cell in anaphase (control). The scale bars, 5 μm. (b) Kinetochores depleted of CENP-H showed an aberrant distribution or lack of CENP-E but still contained CENP-C and hBubR1. HEp-2 cells transfected with control (A, C, and E) or CENP-H siRNA (B, D, and F) were fixed and co-stained for CENP-A to show the position of the kinetochores (left column) and for the kinetochore proteins CENP-C (A,B), hBubR1 (C,D), and CENP-E (E,F) (second column). Chromosomes were stained with ToPro3 (third column). The right column displays the overlay of all 3 stainings. Some kinetochores of misaligned chromosomes in CENP-H deficient cells, which were stained against CENP-C (B), hBubR1 (D), or CENP-E (F), are indicated by arrowheads for comparison. The spindle poles are indicated with arrows. For each of the 3 kinetochore proteins, 150 CENP-H depleted cells at 3 different time points after incubation (24, 48, and 72 h) were analysed. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

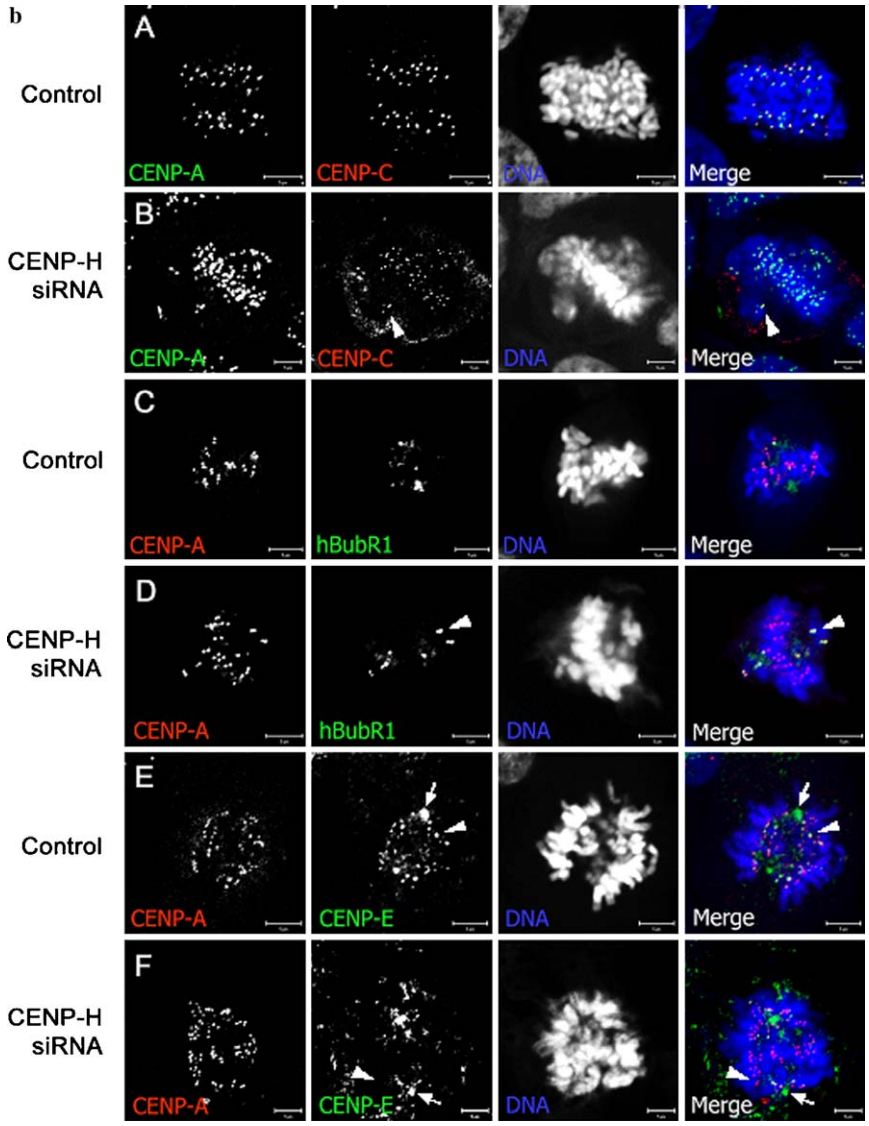

Fig. 3 (*continued*)

an abnormal localisation of CENP-C at the cell periphery. The total protein amount of CENP-C did not change in CENP-H depleted cells (see Fig. 2D). However, CENP-H depleted human kinetochores showed a decreased CENP-C protein level at kinetochores compared to control cells. To quantify the amount of CENP-C at human kinetochores, we determined the background corrected fluorescence intensity of rhodamine linked to a secondary antibody detecting an anti-CENP-C antibody. Using the Metamorph software, we analysed 445 kinetochores in eleven control cells yielding a relative fluorescence intensity set to $100 \pm 1\%$ (Fig. 4c-A). When determining the fluorescence intensity of rhodamine at 364 kinetochores in ten CENP-H depleted cells (72 h after RNAi transfection), we observed a relative fluorescence intensity of $72 \pm 1\%$. This suggests that a reduced amount of CENP-C was incorporated into CENP-H depleted kinetochores. This effect, however, is much smaller in human (-28%) than in chicken cells (-88% [44]). Nevertheless, this result indicates that, as in chicken, strong reduction of CENP-H influences the binding of CENP-C to kinetochores also in human cells.

As described above, we did not observe mitotic arrest in CENP-H knocked-down cells despite appearance of misaligned chromosomes. This led us to the assumption that checkpoint function might be disturbed. Due to differences in mitotic progression in CENP-H depleted human and chicken cells, we analysed the localisation of the checkpoint protein hBubR1 in control and CENP-H deficient cells. hBubR1 is present at the kinetochores until they are correctly aligned in the metaphase plate and connected to spindle microtubules. In this way, together with other checkpoint proteins, hBubR1 grants the "wait-for-anaphase" signal [49,50]. In control cells, we found that hBubR1 was correctly localised at the kinetochores of not aligned chromosomes (Fig. 3b-C). In human cells lacking CENP-H, the protein was still present at kinetochores of misaligned chromosomes (Fig. 3b-D). The protein level

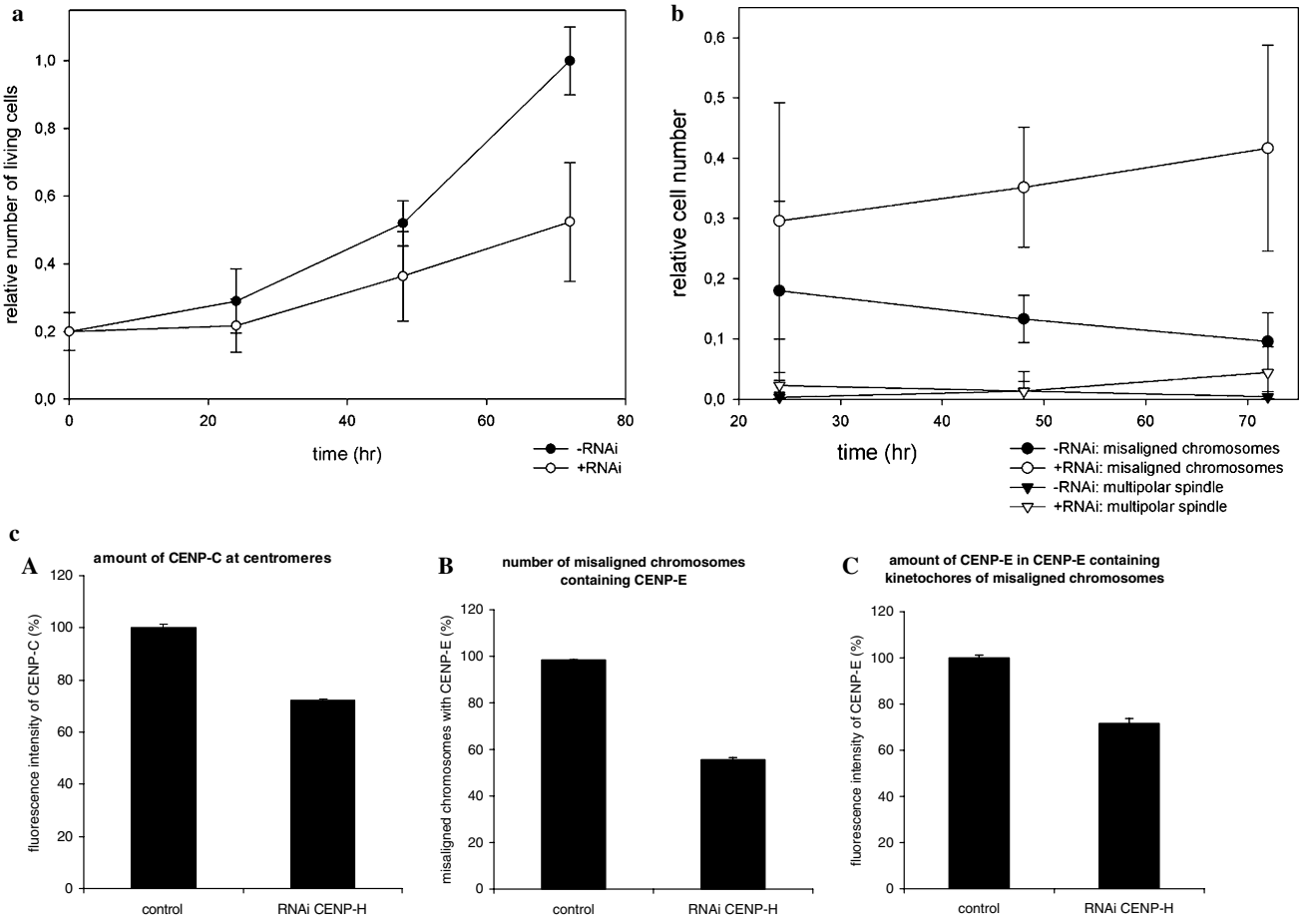

Fig. 4. (a) Depletion of CENP-H resulted in a decreasing growth rate. Control (closed circles) or siRNA treated (open circles) HEp-2 cells were counted after 24, 48, and 72 h (x axis) incubation in a Neubauer chamber using Trypan blue discrimination. The number of living cells (y axis) was quantified in 5 independent experiments. (b) CENP-H deficient cells displayed an increased number of aberrant mitotic phenotypes such as misaligned chromosomes and multipolar spindles. Control (closed labels) and siRNA treated (open labels) HEp-2 cells were fixed after 24, 48, and 72 h incubation (x axis), stained for spindle microtubules, centromeres, and DNA, and analysed in the CLSM. The cell numbers which show misaligned chromosomes (circles) and multipolar spindles (triangles) were quantified and are displayed relative to the number of all cells observed (y axis). For each time point, 450 cells were counted in six independent experiments. (c) CENP-H deficient kinetochores contained a decreased amount of CENP-C and CENP-E and about half of the misaligned chromosomes totally failed to recruit CENP-E. Control or siRNA treated HEp-2 cells were fixed after 72 h incubation and co-stained for CENP-A and for the kinetochore proteins CENP-E and CENP-C, respectively. To quantify the amount of CENP-C (A) or CENP-E (B,C) at each centromere locus, the fluorescence intensity analysis (see text) was performed using Metamorph Offline 6.1r4 software. (A) The CENP-H deficient kinetochores contained only $72 \pm 1\%$ CENP-C compared to untreated control cells. (B) In untreated control cells, $98 \pm 0.2\%$ of the kinetochores of misaligned chromosomes contained CENP-E. In CENP-H deficient cells, this number was reduced to $56 \pm 1\%$. (C) In those kinetochores of misaligned chromosomes which contained CENP-E, the amount of CENP-E was reduced to $72 \pm 2\%$ in CENP-H deficient cells compared to untreated cells.

of hBubR1 was not changed in CENP-H depleted cells as shown by Western blot analysis (see Fig. 2F). Therefore, also at CENP-H depleted kinetochores, at the BubR1 level the mitotic checkpoint seems to be assembled in a correct manner.

When the checkpoint is correctly assembled at CENP-H depleted kinetochores, why then does it not inhibit cell cycle progression until all chromosomes are properly aligned? Recent findings indicate that the checkpoint cannot be established or maintained without the kinetochore-associated microtubule motor protein CENP-E [37,43,50]. The association of CENP-E with hBubR1 stimulates its kinase activity which is required for prevention of premature anaphase in the presence of unattached kinetochores [37]. To find out if CENP-E is still present to activate hBubR1 kinase, we studied the localisation of CENP-E at kinetochores lacking CENP-H. During mitosis, we could detect CENP-E at kinetochores and at the spindle poles in control cells (Fig. 3b-E). CENP-E strongly accumulated on misaligned chromosomes while it was much weaker on kinetochores of aligned chromosomes. In CENP-H depleted cells, the spindle poles were still stained with CENP-E, but most of the protein was detected within the cyto-nucleoplasm in an abnormal pattern (Fig. 3b-F). The amount of CENP-E in CENP-H depleted cells, however, did not change in comparison to control cells, only its localisation was altered (as shown by Western blot analysis, see Fig. 2E). We identified misaligned

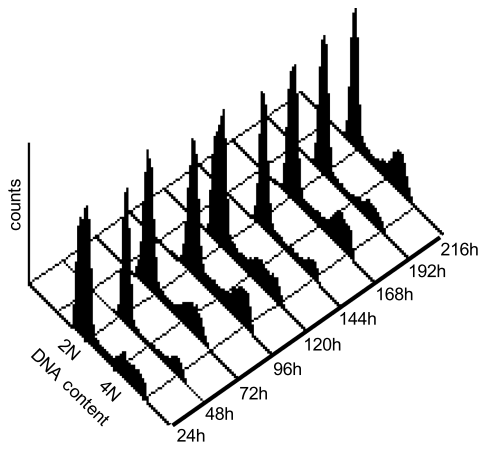

Fig. 5. Cell cycle analysis revealed no mitotic arrest in CENP-H deficient cells. In five different experiments, siRNA treated HEp-2 cells were analysed by FACS after 24–216 h incubation. In each measurement, 50,000 gated cells were counted which were stained with propidium iodide for total DNA detection (linear scale).

chromosomes by CENP-A and DNA staining (as an example, see Fig. 3b-E and b-F) and counted how many of them contained CENP-E at their kinetochores. In control cells, CENP-E was present at kinetochores in 98 ± 0,2% of unaligned chromosomes. After CENP-H depletion, only 56 ± 1% kinetochores of misaligned chromosomes contained CENP-E (after 72 h; Fig. 4c-B). In those misaligned chromosomes containing CENP-E at their kinetochores, we quantified the amount of CENP-E ($n = 150$ kinetochores of nine control cells) by measuring the fluorescence intensity of FITC-labelled secondary antibodies detecting anti-CENP-E antibodies. In CENP-H depleted cells (96 kinetochores of nine cells, after 72 h), we observed a reduced amount of CENP-E of 72 ± 2% (Fig. 4c-C) in the kinetochores of misaligned chromosomes. Thus, we found that slightly less than half of the CENP-A marked kinetochores of misaligned chromosomes totally lacked CENP-E during mitosis and those kinetochores containing CENP-E showed a reduced CENP-E level. Since obviously CENP-H depleted kinetochores often failed to recruit CENP-E, we speculate that BubR1 kinase might not be sufficiently activated. This would possibly lead to a disturbed "wait-for-anaphase" signal with the consequence that the cells could not be arrested in mitosis despite of misaligned chromosomes.

From our data we conclude that CENP-H depletion leads to disturbed kinetochore function during mitosis in human HEp-2 cells.

Discussion

In order to identify and characterise potential kinetochore protein interdependencies and to understand the assembly pathway for the human inner kinetochore, we studied the function of CENP-H in human HEp-2 cells by RNAi knockdown. Several isoforms of CENP-H exist in human cells and our siRNA was able to block each of the isoforms. However, in HEp-2 cells we only found the BF245236 form being present. The knockdown of CENP-H to less than 5% after 72 h of incubation led to the establishment of several abnormal phenotypes as also found in chicken DT40 cells [32,44]: misaligned chromosomes, multipolar spindles, and strongly condensed chromosomes as an indicator of apoptosis. In contrast to chicken, however, in human cells we found no monopolar spindles and observed no metaphase arrest. In addition, chicken DT40 cells showed chromosome shapes (micronuclei and apoptotic bodies) which we did not observe in human HEp-2 cells. From the strongly distorted phenotypes we conclude that CENP-H has an important function in the human inner kinetochore which obviously cannot be (fully) taken over by other inner kinetochore proteins (such a functional replacement was discussed by Fukugawa et al. [44] for CENP-C and CENP-H in chicken).

As a second step, we studied CENP-C localisation in CENP-H deficient cells. In chicken DT40 cells, CENP-H and CENP-I are required for centromere localisation of CENP-C [32,44]. This relates to our findings in human cells: the amount of CENP-C was decreased by 28% at human kinetochores which nearly completely lack CENP-H while it was much more strongly decreased in chicken cells. It could well be, however, that even very low levels of CENP-H are still sufficient to recruit CENP-C to kinetochores and that only a complete CENP-H knockout would abrogate CENP-C recruitment as in chicken.

Furthermore, CENP-H depleted chicken DT40 cells showed metaphase arrest resulting in tetraploidic cells (8N) after 72 h, indicating that the mitotic checkpoint was disrupted after 72 h [44]. In contrast, in human RNAi treated CENP-H knockdown cells, we did not observe mitotic arrest and CENP-H deficient cells proceeded through the cell cycle despite of misaligned chromosomes. This indicates that CENP-H has little or no impact on mitotic progression in human cells.

The outer kinetochore protein ZW10 did not localise to the kinetochore in chicken cells indicative of a disruption of the kinetochore structure and a loss of mitotic checkpoint function in CENP-H depleted chicken DT40 cells [44]. Analysing the human checkpoint protein hBubR1 in CENP-H depleted human cells, we found that it localised at the kinetochore of misaligned chromosomes in the same way as in control cells with CENP-H, indicating that the mitotic checkpoint is functional at the hBubR1 level.

Then, in CENP-H depleted human cells we studied the kinetochore-associated microtubule motor protein CENP-E which interacts with hBubR1 [38,39] and stimulates directly its kinase activity [40,41]. In CENP-H depleted human cells, we observed that CENP-E localised only to about one half (56%) of the kinetochores of not correctly aligned chromosomes and that the protein level of CENP-E at these CENP-E containing kinetochores of misaligned chromosomes was reduced (to 72%) indicating that CENP-E binding to CENP-H depleted kinetochores is distorted. A reduced or lacking CENP-E level at the

kinetochores might lead to a reduced hBubR1 activation [42,50] and thus a mis-regulated "wait-for-anaphase" signal, with the consequence that the cells progress into anaphase despite misaligned chromosomes.

Furthermore, we found that CENP-H depleted cells pass throughout mitosis with an increasing cell number having misaligned chromosomes which after a few cell cycles lead to cell death as indicated by a decreasing number of living cells. A potential interpretation is that the reduced number of kinetochores which are associated with CENP-E and which additionally contain a reduced CENP-E level might not generate the correctly regulated "wait-for-anaphase" signal to arrest cells in mitosis until each kinetochore is properly attached to spindle microtubules. Our results agree with findings of Tanudji et al. [42] who observed that HeLa cells with reduced levels of CENP-E showed mitotic delay but did not prevent anaphase onset in the presence of a few unaligned chromosomes. Our observations are also in line with results obtained for CENP-I depleted human cells: Liu et al. [34] concluded that the collective output from many unattached kinetochores is required in these cells to reach a threshold signal of "wait-for-anaphase" to sustain a mitotic arrest. Their cytological and live-cell analyses of CENP-I deficient cells showed that many chromosomes attached to microtubules and congressed normally, although in each cell several chromosomes failed to align at the metaphase plate. Despite the lack of CENP-E at many kinetochores, the kinetochore attachment to and the chromosome movement along the spindle microtubules could be grossly adopted by other motor proteins like dynein/dynactin granting the segregation of the sister chromatids.

We noticed similar phenotypes in human cells for RNAi knockdown of CENP-H, CENP-I, and CENP-E specified by (i) no mitotic arrest, (ii) reduced number of kinetochores associated with CENP-E, and (iii) the progression to anaphase despite the presence of unattached chromosomes [34,42]. This might hint at a particular connection between these three kinetochore proteins which might be realised by direct or indirect binding. This is supported by the (preliminary) finding that CENP-H might interact with CENP-I [27]. On the other hand, the checkpoint protein hBubR1 is not affected by the depletion of any of these three kinetochore proteins [34,42].

Acknowledgments

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