Expanded View Figures

Figure EV1. Localisation properties of the various tethering constructs.

- A, B HeLa cells expressing the indicated HP1α fusion constructs, stained with Hoechst 33342 and immunostained for CENP-C after pre-extraction. Line scan showing the expressed HP1α construct and CENP-C (i); the expressed HP1α construct only (ii); CENP-C only (iii). Scale bars, 5 µm.
- C Alignment of the CENP-B DNA-binding domain protein sequences from various species. Multiple sequence alignment was performed using Clustal Omega and edited with Aline for display. The following species were used for the alignment: *Homo sapiens, Mus musculus, Cricetulus griseus, Rattus norvegicus, Schizosaccharomyces pombe* (CENP-B homolog proteins 1 and 2), *Ornithorhynchus anatinus, Bos taurus, Cavia porcellus, Macaca mulatta* and Pediculus humanus.
- D Structure of the CENP-B DNA-binding domain is shown as a ribbon diagram interacting with DNA (PDB code 1HLV). Mutated residues are shown in stick.
- E HeLa cells expressing the indicated HP1α fusion constructs, stained with Hoechst 33342 and immunostained for CENP-C. Scale bar, 5 μm.
- F Immunoblot of HeLa cell lysates transfected with the indicated HP1α fusion constructs. HP1α fusion constructs were detected with anti-GFP antibody and αtubulin was used as a loading control.

Α			В		
	CB-EY-HP1a			CB-EY-HP1a	
2	CEMP-C CB ^{mut} -EY-HP1α CENP-C	0.0 0.8 0.6 0.4 0.2 0.0 0 0 0 0 0 0,50µm		CB ^{mut} -EY-HP1α ii CENP-C	1.0 0.8 0.6 0.4 0.0 0.0 0.82µm 1.57
3	EY-HP1α CENP-C	1.0 0.8 0.6 0.4 0.2 0.0 0.50 µm	3	EY-HP1α CENP-C	1.0 0.8 0.6 0.4 0.2 0.0 0 0.75 µm 1.51
C CENPB_HUMAN CENPB_MOUSE CENPB_FAI CENPB_FAI CENPB_FAI CENPB_SCHPO2 CENPB_SCHPO2 CENPB_CAVPO CENPB_CAVPO CENPB_MACMU CENPB_PEDHC	1 10 MG . PKR ROLTFRESS MG . PKR OLTFRESS MG . PKR OLTFRESS MG . PKR OLTFRESS MG . PKR OLTFRESS MG . PKR OLTFRESS . A GPK ROLTFRESS . A GPK ROLTFWESS . A GPK ROLTFWESS . A GPR ALERN FPEN.L MTSVKR KAITIETKL	20 30 1 0 E V E E N P. D L R K G E I A F 1 0 E V E E N P. D L R K G E I A F 1 0 E V E N P. D L R K G E I A F 1 0 E V E N P. D L R K G E I A F 1 0 E V E N A T K R K G E A F 1 0 E V E N P. D L R K G E A F 1 0 E V E N P. D L R K G E A F 5 P Q E R K K N P. D L G R G E D R F V I K M L E S G A K K S D V C	A RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: SWFEELFNKLSSGE RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: RFN PPSTLS: P RFN PPSTLS: P RFN PPSTLS: P RFN PPSTLS:	50 60 L K N K RA I LA SE R K YG L K N K RA I LA SE R K YG L K N K RA I LA SE R K YG L K N K RA I LA SE R K YG L S P K YS Y LD DG L S P K YS Y LD DG S S S S S S S S S S S S S S S S S S S	70 75 75 75 75 75 75 75 75 75 75 75 75 75
CENPB HUMAN CENPB MOUSE CENPB CHIGH CENPB SCHIGH CENPB SCHPO2 CENPB SCHPO2 CENPB ORNAN CENPB CAVPO CENPB MACMU CENPB MACMU CENPB MACMU	& & & & ØØ ØØ K LEG LL I AWF QO IFAA K K EG LL I AWF QO IFAA K LEG LL I AWF QO IFAA K EG LI AWF QO IFAA L DNA VY EN LOG REVE K EG LI AWF QO IFAA K LEG LI AWF QO IFAA K EG LI AWF QO IFAA K EG LI AWF QO IFAA K EG LI AWF QO IFAA K LEG LI AWF QO IFAA K EG LI AWF QO IFAA K LEG LI AWF QO IFAA K EG LI AWF QO IFAA	1000 1000 GLPVK 1 LNEKALRIAE GL	10	H H H H H V V S C S V A H H V V A S C S V A H H V V A S C V T H H H V V A S C V T H H H H V V A S C V T H H H H V V A S C V T H H H H H V V A S C V T H H H H H V V A S C V T H H H H H V V A S C V T H H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H H V V S C S V A H H H V V S C S V A H H H V V S C S V A H H H V V S C S V A H H H V V S C S V A H H H V V S C S V A H H H V V S F T B V V S C S V A H H V V S C S V A H H H V V S C S V A H H V V S C S V S V S V S V S V S V S V S V	40 750 154 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 154 158 155 158 155 158 155 158 155 158 153 158 153 158 153 158 153 158 153 158 154 158 154 157 153 158 154 158
CENPB_HUMAN CENPB_MOUSE CENPE_CRIGR CENPE_RAT CENPB_SCHPO1 CENPB_SCHPO1 CENPB_SCHPO1 CENPB_SCHPO1 CENPB_SCHPO1 CENPB_MCMU CENPB_MACMU CENPB_MACMU	159 A G P A A A G P A A A G P A A A G P A A A S P A A S P A A S P A A S P A A A S P A A			D	
CB-EY-HP1	lα CENP-C	DNA			
CB-EY-HP1	la ^{V22M}				N120
CB-EY-HP1	a ^{1165E}			F kDa 75- 58-	H ^{05E} M ^{114A} E ^{HP3P¹⁰} anti GFP
	-W174A			58-	anti α-Tubulin

Figure EV1.

Figure EV2. Constitutive retention of HP1-bound CPC results in H3S10 phosphorylation in interphase that is sensitive to low concentrations of ZM447439.

- A, B HeLa cells expressing the wild-type HP1α (1) or mutated HP1α^{W174A} (2) tethering construct at the indicated stages, stained with Hoechst 33342 and immunostained for Aurora B and α-tubulin. The image brightness of the EYFP channel was scaled individually to optimise the clarity of the protein localisation, but in both examples, the CB-EY-HP1α^{W174A} was expressed at higher levels. Scale bars, 5 µm.
- C, D HeLa cells expressing the wild-type HP1α tethering construct treated with either DMSO (1) or with 0.5 μM ZM447439 (2) for 60 min before fixation, stained with Hoechst 33342 and immunostained for H3S10ph and α-tubulin (C) or Aurora B and H3S10ph (D). Scale bars, 10 μm.



Figure EV2.





Figure EV3. Live cell imaging demonstrates that the H3S10ph signal in CB-EY-HP1 α expressing cells persists throughout interphase.

A, B Stills of live cell imaging movies using CF640R-labelled Fabs against H3S10ph in HeLa cells expressing the indicated construct. Images were acquired with a 100× objective every 10 min (A) or with a 60× objective every 30 min (B) with five z sections every 2 µm. Scale bars, 5 µm.

Figure EV4. Live cell imaging demonstrates the robustness of the H3S10ph foci in synchronised G₂ cells that are sensitive to low concentrations of ZM447439.

A HeLa CDK1-as cells were transfected with EY-HP1 and treated 24 h after transfection with either 10 μM 1NM-PP1 (+1NM-PP1) or DMSO (-1NM-PP1) for 20 h. Cells were treated with 0.5 μM ZM447439 for 60 min before fixation, stained with Hoechst 33342 and immunostained for Aurora B and H3S10ph. Scale bar, 5 μm.

B Stills of a live cell imaging movie using Cy5-labelled Fabs against H3S10ph in HeLa CDK1-as cells treated with 10 μM 1NM-PP1. Images were acquired every 6 min with five z sections every 1 μm. Scale bars, 5 μm.





Figure EV5. Time difference between the robust appearance of H3S10ph and H3T3ph seems to be smaller in HP1 α + HP1 γ double KO cells.

- A Immunoblot of the indicated HP1 KO cell lines using anti-HP1 α , HP1 β or HP1 γ antibodies and GAPDH was used as a loading control.
- B Stills of live cell imaging movies using Cy5-labelled Fabs against H3S10ph in HeLa wild-type or HP1α and HP1γ double KO cells. Brightness of the far red channel was adjusted individually (0.65% difference), to account for slightly higher amount of loaded Fab fragments in the wild-type cell. Images were acquired every 6 min with five z sections every 1.2 µm. Scale bars, 5 µm.
- C HeLa wild-type (1), or HP1α and HP1γ double KO (2) cells were stained with Hoechst 33342 and immunostained for H3S10ph and H3T3ph. Scale bar, 5 μm.
- D Stills of live cell imaging movies using Alexa488-labelled Fabs against H3S10ph and CF640R-labelled Fabs against H3T3ph in HeLa wild-type or HP1 α and HP1 γ double KO cells. Brightness of the far red channel was adjusted individually (0.76% difference), to account for slightly higher amount of loaded Fab fragments in the HP1 α and HP1 γ double KO cell. Images were acquired every 10 min with five z sections every 1.2 μ m. Scale bars, 5 μ m.



