1 **Intricate structure of the interphase chromocenter revealed by the**
2 **analysis of a factor involved in species formation**

3 Natalia Y. Kochanova[1], Tamas Schauer[2], Grusha Primal Mathias[3], Andrea Lukacs[1], Andreas
4 Schmidt[4], Andrew Flatley[5], Aloys Schepers[5], Andreas W. Thomae[3] and Axel Imhof[1,4*]

5 [1]Biomedical Center, Chromatin Proteomics Group, Department of Molecular Biology, Faculty of
6 Medicine, Ludwig-Maximilians-Universität München, Großhaderner Strasse 9, 82152 Planegg-
7 Martinsried, Germany.

8 [2]Biomedical Center, Bioinformatics Core Facility, Ludwig-Maximilians-Universität München,
9 Großhaderner Strasse 9, 82152 Planegg-Martinsried, Germany.

10 [3]Biomedical Center, Core Facility Bioimaging, Ludwig-Maximilians-Universität München,
11 Großhaderner Strasse 9, 82152 Planegg-Martinsried, Germany

12 [4]Biomedical Center, Protein Analysis Unit, Faculty of Medicine, Ludwig-Maximilians-Universität
13 München, Großhaderner Strasse 9, 82152 Planegg-Martinsried, Germany.

14 [5]Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH),
15 Institute for Diabetes and Obesity, Monoclonal Antibody Core Facility and Research Group
16 Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

17

18 * To whom correspondence should be addressed. Tel: +49 89 218075420; Fax: +49 89 218075410;
19 Email: imhof@lmu.de

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ABSTRACT

In higher eukaryotes centromeres often coalesce into a large intranuclear domain called the chromocenter. Chromocenters are important for the organization of pericentric heterochromatin and a disturbance of their formation results in an upregulation of repetitive elements and causes defects in chromosome segregation. Mutations in the gene encoding for the centromere associated *Drosophila* speciation factor HMR show very similar phenotypes suggesting a role of HMR in chromocenter architecture and function. We performed confocal and super resolution microscopy as well as proximity based biotinylation experiments of HMR and its associated protein HP1a to generate a molecular map of HMR and HP1a bound chromatin. Our work reveals an intricate internal structure of the centromeric chromatin region, which suggests a role of HMR in separating heterochromatin from centromeric chromatin.

INTRODUCTION

In many eukaryotes pericentromeric and centromeric chromatin of multiple chromosomes cluster in interphase to form a nuclear domain, the so-called chromocenter (1-3). The chromocenters are composed of pericentromeric heterochromatin and CenpA containing centromeric chromatin, which are both rich in repetitive DNA and evolutionarily highly dynamic (4, 5). They form and are held together by multiple components ranging from RNA transcribed from the repeats (6) over DNA binding factors (7), protein-protein interactions (8) to histone posttranslational modifications (9). Interference with chromocenter formation results in an upregulation of transposable elements, mitotic defects and the formation of micronuclei (7, 8). Despite their functional importance, the centromere as well as the pericentromeric repeats are highly divergent with regard to size, sequence and protein composition even in very closely related species (4, 10, 11). This rapid divergence of centromeric sequences is thought to be accompanied by an adaptive evolution of centromere binding proteins to counteract a meiotic drive, which would otherwise result in the potentially deleterious expansion of centromeric repeats (5, 12). This hypothesis is supported by the fact that many of the gene products that result in hybrid incompatibility are either proteins that bind to the centromeric or pericentromeric heterochromatin (13-16) or RNA molecules that localize in this region (17, 18). One of the best characterised hybrid incompatibility factors is the Hybrid male rescue protein HMR (19). HMR interacts with the heterochromatin protein HP1a and co-localizes with the centromere-specific H3 variant dCenpA (CID) in *Drosophila* cell lines and imaginal disc cells (13). HMR binding is also observed at several euchromatic sites where it colocalizes with known boundary factors (20). Interestingly, the intracellular localization of HMR varies among different tissues. In interphase cells of larval brains it is primarily found at pericentromeric heterochromatin (15, 21, 22) whereas it also associates with telomere structures on salivary gland polytene chromosomes (13, 22). Mutations of *Hmr* results in an upregulation of transposable elements (13, 23) and an increase in mitotic defects (13). As this phenotype is very similar to defects upon knockdown of nucleoplasmin (Nlp), which interacts with HMR and plays a role in centromere clustering (8, 13), we wondered about HMR's role in chromocenter architecture. To investigate the intricate structure of the *Drosophila* chromocenters we performed confocal and super resolution microscopy using antibodies directed against HP1a,

1   HMR and dCenpA and determined the proteome in proximity of HMR and HP1a using APEX2 based

2   proximity biotinylation. Our results suggest that HMR is located at boundaries between HP1a

3   containing heterochromatin and centromeric or transcriptionally active chromatin. Besides the

4   proximity to heterochromatic and known centromeric factors we also observe a close proximity of

5   HMR to nucleolar proteins, transcription factors, nuclear pore components and the condensin and

6   cohesin complex. These findings suggest an important role of HMR in orchestrating the formation of

7   the evolutionarily very dynamic chromocenter domain. As a consequence, the differential regulation of

8   HMR in different species of *Drosophila* results in complex mitotic defects in hybrid animals containing

9   two different and separately evolved genomes.

10   **RESULTS**

11   **HMR and dCenpA form an interdigitated centromeric network**

12   To confirm the previously detected centromeric localization of HMR in *Drosophila* cells with another

13   antibody, we performed immunofluorescent staining using a FLAG antibody in a cell line where HMR

14   is endogenously tagged with the FLAG epitope at the C-terminus using CRISPR/Cas9 (20).

15   Consistent with our previous results (13) most of the FLAG signals co-localize with the signal we

16   obtained when using an anti-dCenpA antibody, which confirms the close proximity of HMR and

17   centromeric chromatin during interphase (Figure 1A). Notably, the quantitation of more than 120

18   interphase centromeres indicated that 57% of all centromere foci overlap with HMR foci. This

19   suggests that the localization of HMR to the centromere is either cell cycle regulated or specific for a

20   subset of centromeres (Figure 1B), which may explain the differences observed when staining HMR

21   in different tissues (13, 15). Another observation that is difficult to reconcile with the clustered staining

22   of HMR in cells is the fact that ChIP-seq data suggest HMR binding along the euchromatic arms often

23   at boundaries between HP1a containing heterochromatin and actively transcribed genes (20). As

24   centromeres are also transcribed (10) and in proximity to pericentromeric heterochromatin, we

25   wondered if HMR might also localize to a putative boundary between these two different chromatin

26   regions. Due to its repetitive nature, pericentromeric as well as centromeric chromatin is difficult to

27   map in ChIP-seq experiments. We therefore investigated the interphase centromere and its

28   surrounding chromatin using super resolution STED microscopy. These experiments revealed that the

29   chromocenter is not homogenously formed by dCenpA containing chromatin but constitutes a

30   structural meshwork of interdigitated dCenpA and HMR proteins (Fig. 1C, Movie S1), which we

31   assume to constitute an intranuclear domain frequently defined as the chromocenter (24). In

32   agreement to previous results (25), this centromeric region is surrounded by large lobes of HP1a

33   containing heterochromatin, which are often bordered by HMR signals (Supplemental Fig. S1, Movie

34   S1). This intricate structure is further confirmed by super resolution microscopy stainings of dCenpA

35   and dCenpC as well as HMR and dCenpC. These co-stainings revealed a substantial colocalization of

36   dCenpC with dCenpA, in contrast to the shifted localization observed between HMR and dCenpC or

37   dCenpA (Fig. 1D).

38   **HMR and HP1a APEX2 fusion proteins localize similarly to the endogenous proteins**

1   To further unravel the details of this architectural meshwork at the chromocenter, we generated

2   stable cell lines expressing HMR and HP1a fused to an engineered ascorbate peroxidase from

3   soybean (APEX2) under a copper inducible promoter to perform proximity biotinylation (Fig. 2A).

4   Upon treatment of APEX2 expressing cells with biotin phenol and hydrogen peroxide a localized burst

5   of diffusible biotin–phenoxyl radicals is generated. These radicals then react with nearby (< 20 nm)

6   electron rich amino acid side chains leading to the biotinylation of neighboring proteins that can be

7   subsequently purified and identified using shot gun mass spectrometry (26-32). We confirmed the

8   expression of ectopic proteins by Western blotting using antibodies against HMR, HP1a and APEX2

9   (Fig. 2B). The APEX2 protein was fused to a double nuclear localization signal to determine the non-

10   targeted nuclear proteome. To perform the biotinylation reaction at physiological protein levels, we

11   used non-inducing conditions for HMR_{AP} expression but induced the expression of $HP1a_{AP}$ (Fig. 2B

12   and C). For the $APEX2_{NLS}$ cell line we added copper sulfate to the medium before the biotinylation to

13   induce the expression of $APEX2_{NLS}$ and to match $HP1a_{AP}$ expression levels. At the expression level

14   used, HMR_{AP} localizes to centromeres, marked by the centromere-specific histone variant dCenpA,

15    $HP1a_{AP}$ occupies a domain in the nucleus, which coincides with endogenous HP1a staining and

16    $APEX_{NLS}$ localizes to the nucleus (Fig.2B), showing a proper nuclear localization of the fusion proteins

17   under the conditions used.

18   **HMR and HP1a APEX2 fusion proteins biotinylate defined nuclear domains**

19   To analyze the localization of the biotinylation by the expressed fusion proteins, we stained the cell

20   lines for APEX2 and biotin after performing an *in situ* biotinylation reaction (Fig. 3A). Consistent with

21   the limited diffusion of the phenoxy radicals generated by APEX2, we observed a strong biotinylation

22   signal colocalizing with the APEX2-fusion proteins (Fig. 3B). Cells that do not express nuclear APEX2

23   or cells that were not treated with biotin-phenol and hydrogen peroxide only showed background

24   biotinylation signals. As the different cell lines expressed different levels of the APEX2-fusion protein

25   and as HP1a has previously been shown to be very dynamic, we adjusted the concentration of biotin-

26   phenol and the duration of hydrogen peroxide treatment to maintain proper localization while

27   achieving a high degree of biotinylation of the neighboring proteome. While HMR_{AP} cells treated for 25

28   minutes with hydrogen peroxide at a high concentration of biotin-phenol (5 mM) showed a highly

29   localized biotinylation (Supplemental Fig. S2), a similar treatment of $HP1a_{AP}$ cells resulted in a

30   broader biotinylation signal even though a reduced biotin-phenol concentration was used (0.5 mM)

31   (Supplemental Figs. S2 and S3). This broader signal was not observed when $HP1a_{AP}$ cells were

32   treated for only 5 minutes with 0.5 mM biotin-phenol (Supplemental Fig. S3).

33   **Proteomic analysis of HMR and HP1a containing domains**

34   Upon *in vivo* biotinylation, we compared the individual proteomes from cell lines expressing HMR_{AP},

35    $HP1_{AP}$ and $APEX_{NLS}$ that were either treated with biotin-phenol and H_2O_2 or with DMSO only (Figure

36   4A). Using label free quantitation and a statistical analysis of the proximity-based proteome from at

37   least 4 biological replicates we identified 406, 300 and 349 proteins that were specifically biotinylated

38   in HMR_{AP}, $HP1a_{AP}$ and $APEX_{NLS}$ expressing cells respectively (Supplementary Tab. S2). A

1   comparative analysis of the isolated proximity proteomes revealed a strong overlap between the

2   HP1a$_{AP}$ and the APEX$_{NLS}$ proteome (71%), which was not the case for HMR$_{AP}$ and APEX$_{NLS}$ (44%).

3   The latter finding may reflect the lower expression level of HMR, the lower mobility and the more

4   specific localization of HMR$_{AP}$ compared to HP1a$_{AP}$ (Fig. 3B). To further characterize the different

5   proteomic composition within the three proximity proteomes we compared the enriched GO terms

6   using the Gene Ontology Consortium tool (http://www.geneontology.org) (Supplementary Tab. S3).

7   Consistent with its known function we found the GO terms such as chromatin silencing, histone

8   modification and positive regulation of chromatin organization for HP1a$_{AP}$. For HMR$_{AP}$, we found the

9   GO terms heterochromatin organization involved in chromatin silencing, telomere maintenance,

10   mitotic sister chromatid segregation and nucleus organization, which were consistent with the

11   observed phenotypes of HMR mutations in *Drosophila melanogaster* (13, 15, 22, 33, 34). This

12   proximity-based proteome also strengthens the hypothesis that HMR localizes in between dCenpA

13   and HP1a containing chromatin as components of both domains are found in proximity of HMR.

14   Proteins that are biotinylated by all three factors were often highly abundant nuclear factors

15   constituting the splicing machinery or structural proteins such as NLP, D1 or Lamin, which are likely to

16   be in close proximity to most nuclear factors. As the long hydrogen peroxide treatment resulted in a

17   lower HP1a$_{AP}$ staining upon biotinylation, we also measured the HP1a$_{AP}$ proximity proteome upon 5

18   minutes of biotinylation. Due to the shorter biotinylation time we identified less specifically biotinylated

19   proteins (122). When comparing the proteins biotinylated after 5 minutes peroxide treatment with the

20   ones isolated after 25 minutes of biotinylation we identified a higher percentage of previously

21   characterized HP1a interactors after 25 minutes of biotinylation (35, 36) (Supplementary Fig. S4)

22   suggesting that a longer biotinylation reaction covers a broader range of HP1a$_{AP}$ containing domains.

23   To identify proteins that specifically localize close to HMR$_{AP}$ and HP1a$_{AP}$ and are not distributed

24   throughout the entire nucleus, we selected proteins that were preferentially biotinylated in the HMR$_{AP}$

25   or the HP1a$_{AP}$ cell line but not or only to a much lesser degree in the APEX$_{NLS}$ line (Supplementary

26   Tab. S2). As the HP1a$_{AP}$ specific proximity proteome showed a large overlap with the proteome in

27   proximity to APEX$_{NLS}$, only very few chromatin associated factors were significantly closer to HP1a$_{AP}$

28   than to APEX$_{NLS}$ (Supplementary Table S1), which prevented us from further analyzing the HP1a$_{AP}$

29   proximity proteome in great depth. For HMR$_{AP}$, however we detected a substantial number of proteins

30   being much closer to HMR$_{AP}$ than to APEX$_{NLS}$ and therefore displayed the proximity-based proteome

31   of HMR$_{AP}$ in the form of a network graph. In this graph individual proteins are displayed as nodes and

32   previously published interactions as edges (Fig. 4C). Besides a large fraction of nucleolar proteins, we

33   find the condensin and subunits of the cohesin complex, a large fraction of nuclear pore proteins,

34   several insulator proteins, as well as a network of transcription factors including the previously

35   identified hybrid incompatibility factor GFZF, as well as nucleosome remodeling factors and histone-

36   modifying enzymes (Fig. 4C).

Cohesin and condensin factors reside in proximity to HMR

38   We were particularly intrigued by the proximity of HMR$_{AP}$ to cohesin and condesin subunits as it has

39   been recently shown that hybrid male flies that have higher levels of HMR show defects in

5

1   chromosome condensation and integrity reminiscent of those observed in cells depleted of

2   condensins or showing abnormal accumulation of cohesins (15). To validate the proximity of HMR

3   with members of the cohesin complex, we compared the ChIP-sequencing profiles of HMR in S2 cells

4   (20), the cohesin subunit Rad21 (vtd) and the condensin subunit CAP-H2 in Kc167 cells (37).

5   Consistent with the proximity labelling result, approximately half (51%) of the HMR binding sites

6   colocalize with binding sites of the cohesin complex and 20% with the CAP-H2 subunit of the

7   condensin complex (Fig. 5A). The analysis of the genomic features of the HMR binding sites that

8   overlap with Rad21 or CAP-H2 demonstrated that the HMR binding sites colocalizing with condensin

9   or cohesin are more frequently found at promoter regions (Fig. 5B, left panel). By classifying

10 chromatin domains using the five-state chromatin model (38) the HMR binding sites that colocalize

11 with the condensin subunit CAP-H2 were more frequently found at active chromatin or HP1a

12 containing heterochromatin (Fig. 5B, right panel).

13 To simulate the hybrid situation where increased levels of the complex containing HMR and LHR are

14 found (13), we compared a HMR ChIP-seq analysis upon overexpression of HMR and LHR (39) with

15 the condensin and cohesin profiles. Upon overexpression we detected 1314 additional HMR peaks,

16 many of them colocalizing with binding sites of CAP-H2 (Fig. 5C). When we aligned ChIP-seq profiles

17 along HMR binding sites (both native and induced), we found, that CAP-H2 ChIP enrichment is

18 greater at HMR sites having high HP1a signals or at HMR sites appearing only upon overexpression

19 (Fig. 5D). Rad21 ChIP enrichment in contrast is non-discriminating for classes of HMR binding.

20 Architectural proteins, such as condensins and cohesins, were shown to cluster at TAD boundaries

21 (37). We therefore wondered, whether HMR binding sites also reside there. Interestingly, HMR

22 binding sites have local minima in TAD separation score (40) (Figs. 5D and E) suggesting that HMR

23 localizes and potentially functions at genomic sites important for the 3D organization of chromatin.

24 **DISCUSSION**

25 Interspecies hybrids are either sterile or lethal due to the incompatibility of their genomes (41, 42) or

26 the incompatibility of the oocyte cytoplasm with a conflicting parental genome. Crosses between the

27 closely related species *Drosophila melanogaster* and *Drosophila simulans* result in hybrid male

28 lethality dependent on the presence of *D. mel* HMR and its interaction partner LHR from *D. sim*. More

29 recently, gfzf, a gene encoding an essential FLWYTCH domain containing protein residing on the 3rd

30 chromosome of *D.sim*, has also been shown to contribute to male hybrid lethality (43). Though the

31 effect of these factors on hybrid male lethality is well described, their molecular function is still largely

32 unknown. HMR has been implicated in several cellular processes such as the silencing of

33 transposable elements (13, 22, 48) or the faithful chromosome segregation and sister chromatid

34 separation (13, 15, 33). HMR forms distinct foci that frequently overlap with the centromere in *D. mel*

35 cell lines as well as in imaginal discs derived from *D.mel* larvae. An *Hmr* transgene carrying a

36 mutation that affects its centromeric localization fails to kill male hybrids, suggesting that HMR's mode

37 of chromatin binding is causally related to the male lethality phenotype (13, 34). Our systematic

38 microscopic analysis reveals that while most of the HMR foci colocalize with dCenpA this is not

39 always the case. This suggests either a cell cycle regulated HMR-centromere interaction or a

1 chromosome specific binding of HMR similar to the SATIII RNA, which has also been implicated in

2 species formation (44) and only associates with chromosomes 2,3 and X (18). The overall localization

3 of HMR may also be dependent on the cell type as we for example do not observe sizeable telomeric

4 localization of HMR in S2 cells, while it is substantial in polytene chromosomes (13). Super resolution

5 microscopy of the HMR containing nuclear domains furthermore revealed that HMR, dCenpA and

6 dCenpC partially overlap within a nuclear domain that is clearly separated from HP1a containing

7 heterochromatin. We interpret these findings as an indication of HMRs role in separating individual

8 chromatin domains.

9 Within many organisms centromeres, pericentromeric heterochromatin and in some cases even

10 telomeres cluster to form a nuclear domain or aggregate called chromocenter (24). These aggregates

11 are frequently found at the nuclear periphery or around the nucleolus. Strikingly, we find HMR in

12 proximity of nucleolar components and members of the nuclear pore suggesting that it might function

13 in positioning the chromocenter within the nucleus after cell division.

14 Besides these structural components of the nucleus, the HMR proximity proteome also reveals its

15 close localization to factors that separate functional chromatin domains. This is consistent with

16 previous ChIP-seq mapping of HMR binding sites, which showed that a subset of HMR peaks border

17 HP1a containing chromatin at promoters of active genes (20). Many of these HMR binding sites also

18 colocalize with a condensin complex, two subunits of which we also find in close proximity to HMR.

19 The proximity of HMR with condensin and cohesin at sites that separate different chromatin domains

20 is consistent with the findings of Blum and colleagues (15) who described the *Hmr* phenotype as

21 reminiscent to perturbations in cohesin or condensin function with frequent anaphase breakages at

22 boundaries between eu- and heterochromatin. The striking discrepancy between the number of HMR

23 binding sites observed in ChIP-seq experiments and the few histologically visible clusters suggest that

24 HMR binding sites cluster *in vivo* at the chromocenter (Fig. 6). Such a role of clustered boundary

25 regions in organizing nuclear 3D structure has also been observed in human as well as *Drosophila*

26 cells (45, 46) suggesting a common mechanism for TAD organization. These domains not only play

27 important function in the regulation of gene expression but also in reassembling the nuclear structure

28 during the mitotic telophase. Increased levels of HMR, which is also observed in hybrids, results in an

29 accumulation of HMR binding at previously unbound CAP-H2 binding sites which may in turn result in

30 the formation of multiple novel clusters and a dysfunctional organization of the nucleus (47).

31 In summary, our results suggest that HMR is, in conjunction with a highly complex molecular

32 machinery, involved in organizing the nuclear 3D structure. A failure of this tight regulation could then

33 interfere with this well-orchestrated and self-organized assembly process leading to a catastrophic

34 outcome such as hybrid lethality.

35

1   **MATERIAL AND METHODS**

2   **Cloning.** APEX fusions were cloned into pMT vector (13), which was cut with XbaI and NotI. GST-

3   APEX was cloned into pGEX-6P-1 vector (13), cut with EcoRI and NotI. Cloning was performed with

4   In-Fusion cloning kit (Clontech). The list of primers is available in the supplementary table S1.

5   **Cell culture, transfection and generation of stable cell lines.** *Drosophila* L2-4 cells (13) were

6   grown in Schneider medium supplemented with 10% fetal calf serum, penicillin and streptomycin at

7   26°C. To generate a stable cell line, 3-4 million of cells were transfected with 2 μg of plasmid mixed

8   with XtremeGENE HP (Roche) transfection reagent according to manufacturer's instructions. After

9   transfection, cells were selected for 3 weeks with Hygromycin B (Invitrogen) at 100 μg/ml. Optional

10   induction of cell lines with 250 μM $CuSO_4$ was performed 12-24 hours before experiments.

11   **APEX proximity biotinylation, nuclear extraction and immunoprecipitation.** For biotinylation cells

12   were grown in roller bottles (Greiner) to density of $5*10^6$ cells/ml. For biotinylation in solution $5*10^8$-

13   10^9 cells were resuspended in 200 ml biotin-phenol/PBS or DMSO/PBS and incubated for 30 min at

14   room temperature (RT). H_2O_2 was added to biotin-phenol treated cells to the concentration of 1 mM

15   and cells were pelleted for 20 min at 250 g. The supernatant was aspirated, and cells were washed

16   three times in quenching solution (10 mM sodium azide, 10 mM sodium ascorbate and 5mM Trolox in

17   PBS) or (untreated cells) in PBS. The last washing step was performed in 15 ml falcons. Nuclei

18   isolation and extraction was performed as in (48) with modifications. All buffers were supplemented

19   with freshly added protease inhibitors. Cells were swelled in 3 packed cell volumes (PCV) (e.g. 2,1

20   ml) of hypotonic buffer (10 mM Tris pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA) on ice for 30

21   min. Cells were centrifuged 10 min 250 g, and the pellet was resuspended in 3 PCV of hypotonic

22   buffer supplemented with 0,2% NP-40. Cells were rotated for 10 min at 4°C for the lysis of plasma

23   membrane. Nuclei were pelleted at 1000 g for 10 min and washed with 2 ml of quenching solution

24   supplemented with protease inhibitors. After the wash nuclei were centrifuged for 10 min with 1500 g

25   and the nuclear pellet was snap-frozen in liquid nitrogen. Nuclei were resuspended in 3 ml Tris-Ex100

26   buffer (10 mM Tris pH 7.6, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA and 10% v/v glycerol) + 1500

27   units Mnase + 1500 units Benzonase + 2mM $CaCl_2$. Chromatin was digested for 20 min at 26°C and

28   the reaction was stopped by addition of EDTA and EGTA to 10 mM on ice. Nuclei were disrupted by

29   Dounce homogenization using 10 strokes with a tight-fitting pestle. For chromatin extraction and

30   solubilization, NaCl (to 600 mM) and detergents (Triton X-100 to 1%, sodiumdeoxycholate (SOD) to

31   0.5% and SDS to 0.1%) were added and chromatin was incubated for 1h at 4°C. Nuclear extracts

32   were cleared by centrifugation 20 min 10000 g and dialyzed for 4 hours at 4°C through 3.5 MWCO

33   Millipore membranes against Tris-Ex100 with detergents and without glycerol, supplemented with 0.2

34   mM PMSF and 1 mM DTT. The obtained nuclear extract was snap-frozen. For streptavidin

35   purification, nuclear extract with freshly added protease inhibitors was mixed with 500 μl of Pierce

36   streptavidin magnetic beads, prewashed two times in Tris-Ex100 buffer with detergents.

37   Immunoprecipitation was performed for 1,5 hours at RT. Beads were washed two times with Tris-

38   Ex100 buffer with detergents (with protease inhibitors), once with 2 M Urea 10 mM Tris, and again

39   twice with Tris-Ex100 with detergents and protease inhibitors.

1   For biotinylation on plates, 800 ml of $5*10^9$ million/ml cells were adhered on 40 15-cm plates for

2   mammalian cells for 1 hour. Cells were incubated in biotin-phenol/PBS (20 plates) or DMSO/PBS (20

3   plates) for 30 min, and H_2O_2 was added to biotin-phenol treated cells to the concentration of 1 mM for

4   5 min. Solution was aspirated and quenching solution (or PBS for untreated cells) was added. Cells

5   were scraped off, washed one more time in quenching solution and subjected to nuclear extraction as

6   described above.

7   **Mass spectrometry.** Beads from APEX pulldowns were washed 3 times with 500 µl of 50 mM Tris

8   pH 7.5, 4M urea, and on-bead digestion was performed. Reduction was completed in 500 µl of 20 mM

9   DTT in 50 mM Tris, 2M urea pH 7.5 with Lys C 450 ng/sample at 27°C for 1h. Alkylation followed with

10 50 mM iodoacetamide for 1 h 25°C, shaking 900 rpm, and was stopped by addition of DTT to 10 mM

11 final concentration. The samples were incubated at 25°C shaking 900 rpm for 2 more hours. 300 µl

12 water was added to reduce urea concentration to 1.5 M, and 1.5 µg trypsin and 2 mM final

13 concentration $CaCl_2$ were added for overnight incubation shaking 900 rpm. In the morning another 1.5

14 µg of trypsin was added, and the sample was incubated for another 4 hours while shaking. After

15 collection of the supernatant, the beads were washed 2 times with 100 µl of 20 mM Tris 50 mM NaCl

16 25% ACN in order to elute loosely-bound tryptic peptides from the beads. Washes were combined

17 with supernatant, evaporated at less than 28°C, and desalted. The second elution of remaining

18 peptides from the beads was performed with 300 µl 0.05% SDS, 0.1% formic acid (FA) at 80°C for 10

19 min. The second elution was evaporated and subjected to HILIC chromatography. After desalting and

20 HILIC both elutions were combined, evaporated and resuspended in 45 µl 0.1% FA. Peptide mixtures

21 were subjected to nanoRP-LC-MS/MS analysis on an Ultimate 3000 nano chromatography system

22 coupled to a QExactive HF mass spectrometer (both Thermo Fisher Scientific) in 2-4 technical

23 replicates (5 µl each). The samples were directly injected in 0.1% FA onto the separating column (120

24 x 0.075 mm, in house packed with ReprosilAQ-C18, Dr. Maisch GmbH, 2.4 µm) at a flow rate of 0.3

25 µl/min. The peptides were separated by a linear gradient from 3% ACN to 40% ACN in 50 min. The

26 outlet of the column served as electrospray ionization emitter to transfer the peptide ions directly into

27 the mass spectrometer. The QExactive was operated in a TOP10 duty cycle, detecting intact peptide

28 ion in positive ion mode in the initial survey scan at 60,000 resolution and selecting up to 10

29 precursors per cycle for individual fragmentation analysis. Therefore, precursor ions with charge state

30 between 2+ and 5+ were isolated in a 2 Da window and subjected to higher-energy collisional

31 fragmentation in the HCD-Trap. After MS/MS acquisition precursors were excluded from MS/MS

32 analysis for 20 seconds to reduce data redundancy. Siloxane signals were used for internal

33 calibration of mass spectra.

34 **Protein MaxQuant search.** The raw data files of APEX pulldowns were analysed with MaxQuant

35 version 1.5.3.12 against dmel-all-translation-r6.08.fasta database from Flybase. All the parameters

36 were set to default except choosing „Match between runs" and LFQ and iBAQ quantitations.

37 Technical replicates were assigned to one experiment (biological replicate).

38 **Data sources.** ChIP-seq datasets were available from GEO with the following accession numbers:

39 GSE86106 (HMR native and HP1a), GSE118291 (HMR induced), GSE54529 (Rad21 and CAPH2).

1   5-state chromatin domains were taken from (43) from which "yellow" and "red" were merged to active

2   domains. Genomic regions (promoters, exons etc.) are based on the Flybase annotation (version

3   r6.17). TAD separation scores derived from http://chorogenome.ie-

4   freiburg.mpg.de/data_sources.html. Genomic coordinates from the dm3 release were converted to

5   dm6 using the liftover tool from UCSC.

6   **ChIP-seq analysis.** Raw reads were aligned to the reference genome (UCSC dm6) using bowtie2

7   (version 2.2.9) and quality filtered by samtools (version 1.3.1). ChIP-seq profiles were created by

8   Homer (version 4.9) and normalized by the number of reads and the corresponding input. Peaks were

9   called by Homer with parameters -style factor -F 2 -size 200 (except for Rad21 -F 4). Peak overlaps

10 were plotted as Venn diagrams using the Vennerable R package (version 3.1.0.9). HMR peaks were

11 sub-grouped by the overlap with CAPH2 or Rad21 peaks. Such peak groups were characterized by

12 genomic features (promoters, exons, etc.) and chromatin domains (active, etc.) by taking the size of

13 the intersecting genomic ranges between the peaks and each feature and dividing by the sum of the

14 intersecting range sizes. ChIP-seq profiles were aligned to the pool of native and induced HMR peaks

15 and visualized as heatmaps or as average composite plots. Heatmaps were ordered by HMR native

16 enrichment and grouped by HP1a enrichment. Figures were created by R base graphics.

17 **Mass spectrometry analysis.** Log2-transformed LFQ values were imputed with the impute.MinProb

18 function (imputeLCMD R package v2.0) with parameter q = 0.05 and normalized by median

19 normalization. Moderated t-statistics were computed by fitting a linear model with empirical Bayes

20 moderation using the limma R package (version 3.34.9). The data were visualized as volcano plots

21 using R graphics. Code is available upon request. GO-term analysis was done using Gene Ontology

22 Consortium (http://geneontology.org) tool excluding redundant GO terms. The protein-protein

23 interaction network was visualized using Cytoscape. Protein-protein interaction data were taken from

24 the STRING database (49) using experiments, databases, gene fusion and co-occurrence as

25 interaction sources and minimum required interaction score of 0.9. Additional interactions were taken

26 from the database Flybase and indicated as dotted lines.

27 **Immunofluorescent staining.** Cells were adhered on coverslips for 30 min, washed with PBS and

28 fixed in 0.3% Triton X-100/3.7% formaldehyde/PBS for 12 min at RT. After wash with PBS cells were

29 permeabilized in 0.25%Triton/PBS on ice for 6 min. Cells were rinsed 2 times and washed again twice

30 with PBS, following blocking in Image-iT FX signal enhancer (Invitrogen) for 45 min at RT. Primary

31 antibodies, diluted in 5% normal goat serum (NGS) (Dianova), were incubated with coverslips 1 h at

32 RT (or at 4°C overnight for high resolution microscopy). Coverslips were washed with 0.1% Triton X-

33 100/PBS and PBS and incubated with secondary antibody in 5% NGS 1 h at RT. Following a wash in

34 0.1% Triton X-100/PBS and 2 washes in PBS, cells were stained in DAPI/PBS (200 ng/mL or 50

35 ng/mL for high resolution microscopy) and washed again in PBS. Cells were mounted in

36 VECTASHIELD (Vector Labs) or ProLong™ Diamond Antifade (Thermo Fisher Scientific), for

37 epifluorescence and confocal or super resolution microscopy, respectively.

1   For biotinylation followed by immunofluorescence on coverslips, 10^6 cells was adhered on coverslips

2   for 30 min, incubated with biotin-phenol/PBS or DMSO/PBS for 30 min, followed by (optional) addition

3   of 1mM H_2O_2 for denoted time. Cells were next washed in quenching solution and subjected to

4   immunofluorescent staining as described above. Biotinylation in solution was performed with 10^6 cells

5   in 200 µl biotin-phenol/PBS or DMSO/PBS and 1 mM H_2O_2, added for denoted time. Cells were

6   pelleted during biotinylation 250 g, resuspended in 200 µl quenching solution, adhered on coverslips

7   for 15 min and processed for IF as described above.

8   Image acquisition was performed on a Zeiss Axiovert 200 epifluorescence microscope with a CCD

9   Camera (AxioCam MR, Zeiss). Confocal microscopy was performed at the core facility bioimaging of

10   the Biomedical Center with an inverted Leica SP8X WLL microscope, equipped with 405 nm laser,

11   WLL2 laser (470 - 670 nm) and acousto-optical beam splitter. Gated-STED Images were acquired

12   with a 100x1.4 objective, image pixel size was 24-25 nm. The following fluorescence settings were

13   used: DAPI (excitation 405 nm; emission 415-470 nm), Alexa Fluor 594 (590 nm; 600-625) and

14   Abberior STAR 635P (635; 645-720). Recording was done line sequentially to avoid bleed-through

15   and channel misalignment because of drift. Signals were recorded with hybrid photo detectors (HyDs)

16   in counting mode. STED and confocal images were deconvolved using Huygens 17.10 p2. All images

17   were processed using ImageJ.

18   **Western blotting of whole cell extracts.** 15 million cells from each cell line were collected, washed

19   two times in PBS and resuspended on ice in 80 µl RIPA buffer with freshly added protease inhibitors

20   and 30 units benzonase. Lysates were left on ice for 30 min, and afterwards 20 µl of 5x Laemmli

21   buffer was added. Lysates were boiled 10 min 96°C before loading on the gel (10 µl).

22   **Antibodies.** For immunofluorescence and western blotting, rat anti-HMR 2C10 antibody (Helmholtz

23   Zentrum München, (13)) was used in dilution 1:25 (or 1:5 for high resolution microscopy); rat anti-

24   dCenpA 7A2 (Helmholtz Zentrum München, (8)) 1:100 (or 1:50 for high resolution microscopy); rabbit

25   anti-dCenpA (Immunofluorescence grade, Actif Motif) 1:500 (or 1:250 for high resolution microscopy);

26   mouse anti-HP1a C1A9 (50) 1:100; anti-APEX2 20H10 (raised in this study, Helmholtz Zentrum

27   München) 1:50; Streptavidin-Alexa555 (Thermo Fisher Scientific) 1:400; rabbit anti-histone H3

28   (Abcam) 1:3000, mouse anti-FLAG (Sigma M2, 1mg/ml) 1:100. Rabbit anti-dCenpC antibody was

29   kindly provided by Christian Lehner and used in dilution 1:5000 (1:1000 for high resolution

30   microscopy).

31   **Antibody generation.** Wistar rats were immunized subcutaneously (s.c.) and intraperitonially (i.p.)

32   with 50 µg of GST-APEX fusion protein dissolved in 500µl PBS, 5 nmol CpG2006 (TIB MOLBIOL) an

33   equal volume of incomplete Freund's adjuvant. 6 weeks after immunization a 50 µg boost injection

34   was applied i.p. and s.c. three days before fusion. Fusion of the splenic B cells and the myeloma cell

35   line P3X63Ag8.653 was performed using polyethylene glycol 1500 according to standard protocols

36   (51). Hybridoma supernatants were tested by solid-phase enzyme-linked immunoassay (ELISA) using

37   the recombinant GST-fusion protein and verified by Western blotting of whole cell extracts from

38   APEX2 fusions-expressing cell lines (Fig. 2B). Hybridoma cell line from specifically reacting

1   supernatants were cloned twice by limiting dilution. Experiments in this study were performed with

2   clone 20H10 (rat IgG2a/κ).

3   **DATA AVAILABILITY**

4   The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium

5   via the PRIDE (52) partner repository with the dataset identifier PXD011310.

16   **CONFLICT OF INTEREST**

17   The authors state no conflict of interest

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1   **TABLE AND FIGURES LEGENDS**

2

3   **Figure 1: (A**) – Staining of S2 FLAG-HMR line (20) with mouse anti-FLAG (Sigma), anti-HMR 2C10

4   and rabbit anti-dCenpA antibodies. (**B**) Quantification of centromeres overlapping with HMR.

5   Experiments were performed in a dCenpA-GFP expressing cell line (53). In total 25 cells and 122

6   centromeres identified using an anti GFP antibody were counted. (**C**) Confocal (top panel) and

7   stimulated emission depletion (STED) microscopy (middle panel) images of dCenpA and HMR. The

8   bottom panel shows a zoom in of the centromeric region (**D**) Plot profiles of the highest fluorescence

9   intensities of STED images of the chromocenter from S2 cells using anti dCenpA, anti dCenpC and

10   anti HMR antibodies. Intensity graphs were built in ImageJ and normalized to one of the maximum

11   peaks.

12   **Figure 2: (A)** APEX fusion constructs used for proximity based labeling of the chromocenter. **(B)**

13   Western blots of whole cell lysates from stable cell lines, expressing HMR$_{AP}$, HP1a$_{AP}$ or APEX$_{NLS}$.

14   Antibodies used for Western blots were anti-HMR 2C10, anti-HP1a C1A9, anti-APEX2 20H10 and

15   rabbit anti-H3 (for loading control). **(C)** Localization of APEX fusions in respective stable cell lines.

16   Antibodies used are the same as for Western blotting in (**B**). For cells with centromere staining

17   (indicated by an asterisk) the inlet shows an approx. 2.3-fold zoom of the nucleus. Shown are the

18   stainings of cells with (induced) or without (uninduced) copper mediated induction of the expression of

19   the corresponding fusion protein.

20   **Figure 3: (A)** Schematic display of a proximity based biotinylation experiment. **(B)** Biotinylation

21   experiment performed for 1 minute with 5 mM biotin-phenol using the HMR$_{AP}$ cell line (top four panels)

22   in the absence of biotin phenol and hydrogen peroxide, the presence of either biotin phenol or

23   hydrogen peroxide or both reagents. The bottom panel shows a treatment of non-APEX expressing

24   L2-4 cells treated with biotin phenol and hydrogen peroxide. For selected cells (indicated by an

25   asterisk) the inlet shows an approx. 2.3-fold zoom of the nucleus. **(C)** Proximity based biotinylation of

26   the HP1a$_{AP}$ and the APEX$_{NLS}$ cell lines using 0.5 mM biotin-phenol. Stainings were performed with

27   anti-APEX 20H10 antibody and anti-Streptavidin Alexa555. Scale bars represent 5 µm.

28   **Figure 4: (A)** Volcano plots of streptavidin pulldowns from extracts prepared from cells expressing

29   APEX$_{NLS}$, HP1a$_{AP}$ and HMR$_{AP}$ either mock treated or treated with biotin phenol and peroxide. Proteins

30   specifically biotinylated in the treated samples are indicated in red. **(B)** Venn diagram of proteins

31   found enriched in the individual purifications. **(C)** Network diagram of proteins specifically enriched in

32   HMR$_{AP}$ but not in APEX$_{NLS}$. Solid lines were provided by the STRING databases using the highest

33   stringency settings (0.9). Dotted lines were manually added based on information from Flybase.

34   **Figure 5 (A)** Peak overlaps of HMR, Rad21 and CAP-H2. **(B)** Distribution of HMR, HMR/Rad21 and

35   HMR/CAP-H2 peaks across different genomic regions and chromatin types. **(C)** Peak overlaps of

36   overexpressed HMR, Rad21 and CAP-H2. **(D)** Heatmaps of HMR (20), overexpressed HMR, HP1a

37   (20), Rad21, CAP-H2 ChIP-seqs (54) and TAD separation score (40). All the peaks are centered at

38   HMR binding sites and clustering is performed as in (20). **(E)** Composite plot of overexpressed HMR,

16

1   Rad21, CAP-H2 ChIP-seq signals and TAD separation score centered to the HMR peaks' position.

2   Peaks that are only detected when HMR is overexpressed are indicated as new.

3   **Figure 6:** Model of organization of *Drosophila* interphase centromere. HMR and dCenpA protein

4   domains are interdigitating, and HMR domains are often bordering dCenpA domains from HP1a

5   domains. Some TAD boundaries, occupied by HMR, might be clustered at the border between

6   centromeric and pericentric chromatins.

7   **SUPPLEMENTARY DATA**

8   **Figure S1:** Confocal microscopy images of L2-4 cells stained with rabbit anti-dCenpA, anti-HMR

9   2C10 and anti-HP1a C1A9 antibodies. The highest intensities graph was built in ImageJ and

10   normalized to one of the maximum peaks.

11   **Figure S2:** Biotinylation experiment performed for 25 minutes using the HMR_{AP}, $HP1a_{AP}$ and $APEX_{NLS}$

12   cell lines. Controls are shown in the right panel. Stainings were performed with anti-APEX 20H10

13   antibody and anti-Streptavidin Alexa555. For selected cells (indicated by an asterisk) the inlet shows

14   an approx. 2.3x zoom of the nucleus. Scale bars represent 5 µm. Exposure for APEX and biotin is

15   indicated in white.

16   **Figure S3:** Time-course biotinylation experiment performed for the $HP1a_{AP}$ cell line. Stainings were

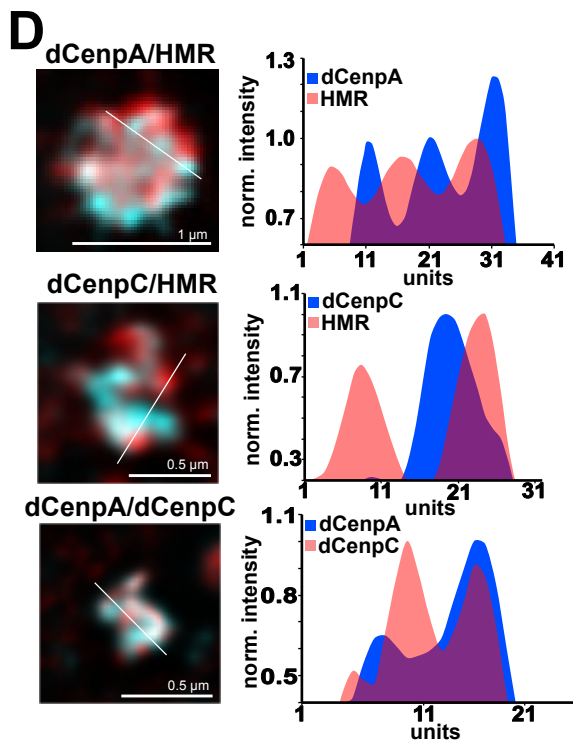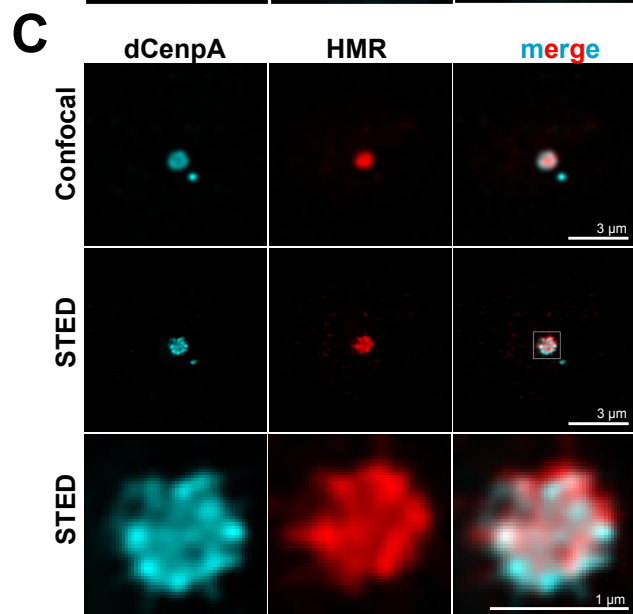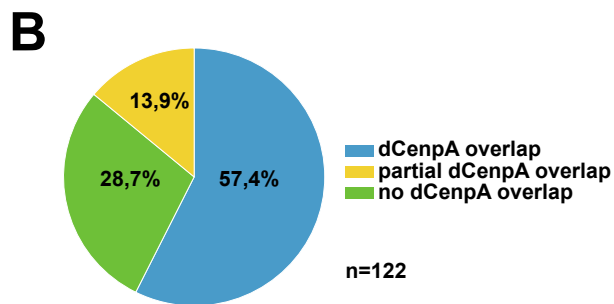17   performed with anti-APEX 20H10 antibody and anti-Streptavidin Alexa555. Scale bars represent 5
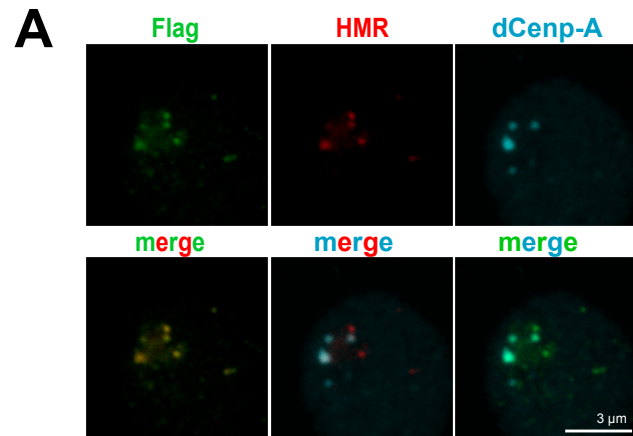
18   µm.

19   **Figure S4:** Volcano plots of streptavidin pulldowns from extracts of cells expressing $HP1a_{AP}$ either

20   mock treated or treated with biotin phenol and peroxide (for 5 and 25 minutes). Previously reported

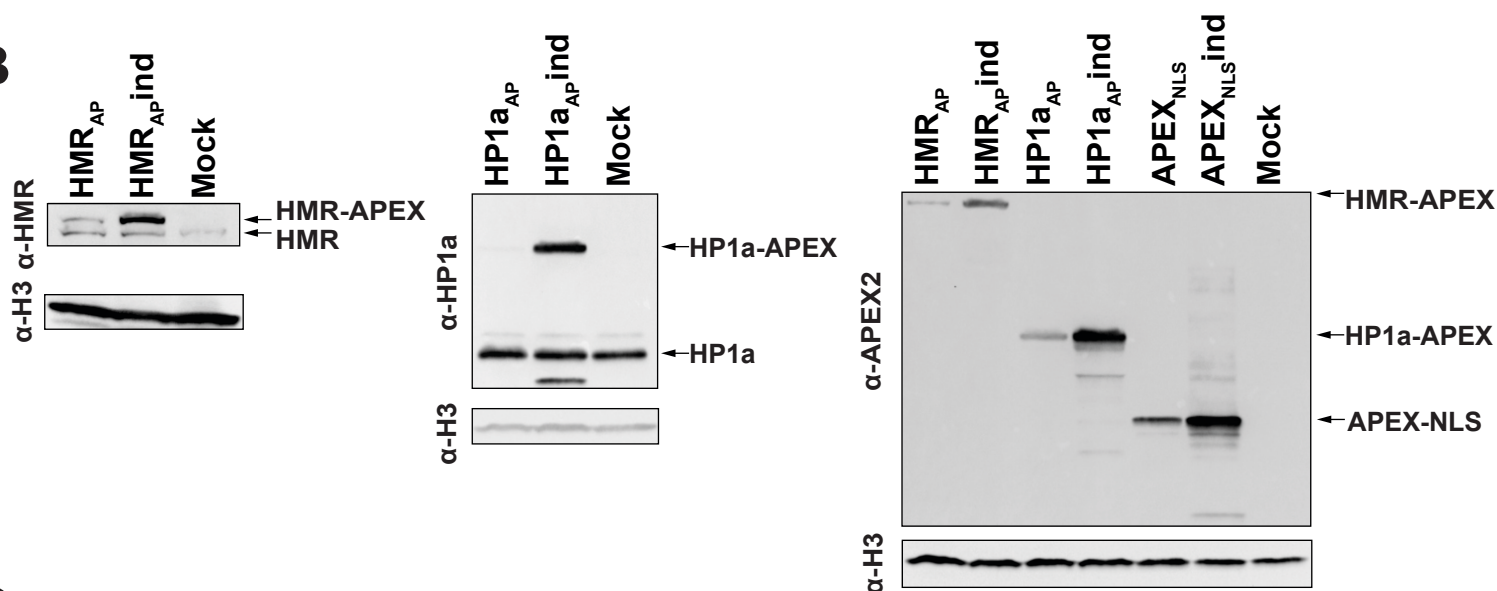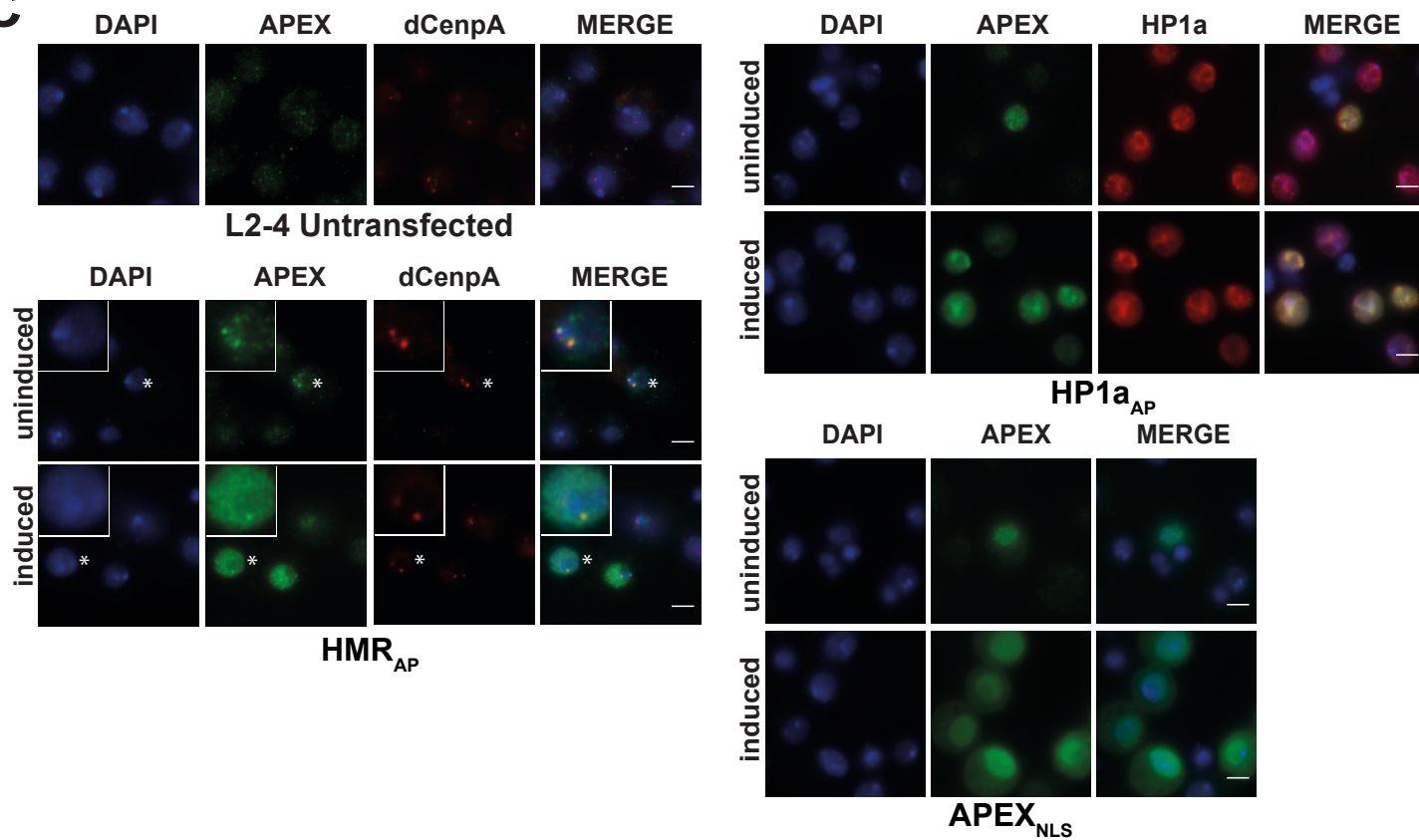21   HP1a interactors, enriched in the pulldowns, are highlighted in green.

22   **Table S1:** List of primers used for cloning of Myc-HMR-APEX, Flag-HA-HP1a-APEX, Flag-HA-APEX-

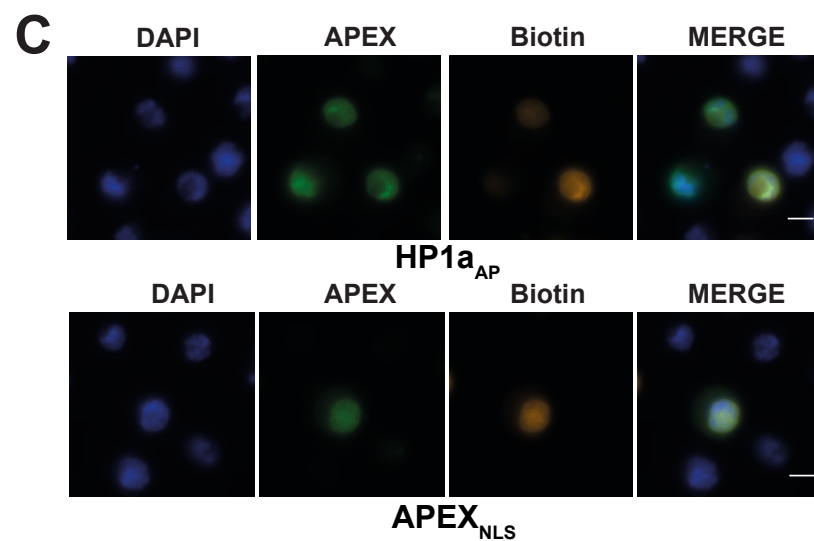23   NLS and GST-APEX constructs.

24   **Table S2:** Lists of proteins, enriched in HMR_{AP}, $HP1a_{AP}$ and $APEX_{NLS}$ pulldowns from treated against

25   untreated cells, as well as proteins enriched in HMR_{AP} and $HP1a_{AP}$ pulldowns against $APEX_{NLS}$.

26   **Table S3:** GO term analysis of proteins enriched in HMR_{AP}, $HP1a_{AP}$ and $APEX_{NLS}$ pulldowns from

27   treated against untreated cells. GO terms found in HMR_{AP} and $HP1a_{AP}$ but not $APEX_{NLS}$ are

28   highlighted in light green.

29   **Movie S1:** 3D reconstruction from confocal HP1a (green) and super resolution STED HMR (red) and

30   dCenpA (blue) microscopy.

31

32

A Flag | HMR | dCenp-A

merge | merge | merge

3 µm

B

13,9%

28,7%   57,4%

- dCenpA overlap
- partial dCenpA overlap
- no dCenpA overlap

n=122

C dCenpA | HMR | merge

Confocal

3 µm

STED

3 µm

STED

1 µm

D

dCenpA/HMR

1 µm

dCenpC/HMR

0.5 µm

dCenpA/dCenpC

0.5 µm

Kochanova et al., Fig. 1

Kochanova et al., Fig. 2

A

Incubation with biotin-phenol → + 1mM H₂O₂ → Quenching free radicals → Microscopy or Nuclear extraction, pulldown and LC-MS

B

Biotin-Phenol	H₂O₂	DAPI	APEX	Biotin	MERGE
−	−				
+	−				
−	+				
+	+				

HMR_AP

| + | + | | | | |

L2-4 Untransfected

C

DAPI	APEX	Biotin	MERGE

HP1a_AP

DAPI	APEX	Biotin	MERGE

APEX_NLS

Kochanova et al., Fig. 3

A

APEX_NLS

HP1a_AP

HMR_AP

y-axis: -log10 p-Value
x-axis: log2(Bio/Con)

B

APEX_NLS

105

30 | 67

147

HMR_AP

206 | 23

63

HP1a_AP

C

Active Transcription

Centromere

Boundary Factors

Basal Transcription

Nucleosome Remodellers

rRNA Maturation Nucleolus

Cohesin

Condensin

Nuclear Pore

A

CAPH2
870
1223
31
177
3120
336
451
Rad21
HMR

B

| | promoter | exon | intron | intergenic |

HMR
HMR/CAPH2
HMR/Rad21
Genome

0.0 0.2 0.4 0.6 0.8 1.0
Fraction

| | active | inactive | polycomb | HP1 |

HMR
HMR/CAPH2
HMR/Rad21
Genome

0.0 0.2 0.4 0.6 0.8 1.0
Fraction

C

CAPH2
801
847
104
549
2887
578
747
Rad21
HMR_ind

D

HMR native   HMR_ind   New   HP1a   Rad21   CAPH2   TAD Sep. Score

-10kb 0 +10kb   -10kb 0 +10kb   -10kb 0 +10kb   -10kb 0 +10kb   -10kb 0 +10kb   -10kb 0 +10kb

1 2.5 4   1 2.5 4   1 2.1 3.2   3.7 6.4   1 1.8 2.7   -0.06 0 0.06

E

ChIP Enrichment
6
5
4
3
2
1

CAPH2
HMR_ind
Rad21

+2kb 0 +2kb

TAD Sep. Score
×10^-2
-8
-12
-16

TAD Sep. Score

+2kb 0 +2kb

Kochanova et al., Fig.5

Kochanova et al., Fig.6