An experimental and computational framework to build a dynamic protein atlas of human cell division

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Essential biological functions of human cells, such as division, require the tight coordination of the activity of hundreds of proteins in space and time. While live cell imaging is a powerful tool to study the distribution and dynamics of individual proteins after fluorescence tagging, it has not yet been used to map protein networks due to the lack of systematic and quantitative experimental and computational approaches. Using the cell and nuclear boundaries as landmarks, we generated a 4D image data-driven, canonical, computational model for the morphological changes during mitotic progression of human cells. We show that this model can be used to integrate the dynamic distribution of 3D concentration data for many mitotic proteins imaged by absolutely calibrated fluorescence microscopy in a large number of dividing cells. Analysis of a pilot data set, containing 28 proteins of interest imaged in dividing cells, allowed us to automatically identify sub-cellular structures and quantify the timing and magnitude of protein fluxes between them, as well as predicting dynamic multi-molecular biological processes such as organelle dis/assembly. Our integrated experimental and computational method enables building a 4D protein atlas of the dividing human cell. As part of the MitoSys and Systems Microscopy consortia, we provide here an

approach that is generic and allows the systematic mapping and mining of dynamic protein localization networks that drive cellular functions.

INTRODUCTION

The specific localization, the timing of particular interactions and the favored binding partners are all crucial to ensure correct function and regulation of proteins and their complexes[1–3]. Moreover, changes in cellular shape and internal structure, which are determined by the activities of proteins, can often feedback on their functions inside the cell[4]. To understand how cells function at the molecular level, it is therefore essential to understand how networks of proteins behave within dynamic cellular boundaries. Mitosis as a fundamental process of life is a good example of a dynamic process orchestrated by a large network of proteins. Within an hour, cells undergo dramatic changes in their morphology and sub-cellular organization. The MitoCheck consortium identified hundreds of essential proteins[5] which need to act in a coordinated fashion in both space and time to drive these changes and respond to them to ensure correct division.

Despite a considerable amount of data on individual mitotic proteins and complexes, our comprehensive understanding of human cell division as a system is limited. To overcome this limitation, the integration of the current knowledge into a single model is necessary. This model can then be used to predict and analyze the dynamic interactions between the molecular parts of the mitotic machinery. Building such a model would require standardized and quantitative data on many different proteins observed in live dividing human cells, as well as the computational tools to integrate and mine such large data sets. Due to the advent of endogenous gene tagging and automated fluorescence correlation spectroscopy (FCS)[6], quantitative 4D protein localization data can now be generated not only in yeast[7], but also in human cells. Although previous computational studies on multiple subcellular localizations have achieved automatic recognition[8,9] and modeling of individual sub-cellular structures either with descriptive models[10,11] or with generative models[12,13], we are still lacking computational methods that can deal with the complexity of dynamic morphological changes of cells and the simultaneous localization of proteins to multiple structures.

Within the MitoSys consortium, we developed a new integrated experimental and computational framework for time-resolved, 3D protein localization analysis within a

dynamically changing cellular context. It integrates the large amount of 3D+time image data acquired by live imaging of fluorescently tagged mitotic proteins by automatic FCS-calibrated confocal microscopy, into a single canonical model of the human dividing cell. This allows both the estimation of subcellular fluxes and the prediction of interactions between all proteins in the model.

RESULTS

Automatic generation of a quantitative 4D protein localization data set

To provide standardized, quantitative data on the dynamic 3D localization of mitotic proteins, we imaged HeLa cell lines, in which such proteins were knocked-in fluorescently (see Online Methods). For each protein, the cell surface and chromosome volume were recorded in separate channels as spatio-temporal landmarks (using either histone H2B-mCherry or SiR-DNA[14] and extracellular dextran-Dy481XL, see Online Methods). To effectively record high-resolution data during mitosis, cells beginning cell division (prophase), were automatically detected in low resolution images using the chromosome signal. They were then imaged by high resolution, confocal microscopy in 3D for one hour until division was completed (**Fig. 1a**). At the end of each 3D mitosis movie, FCS[15] was performed in the mitotic protein channel at six automatically selected positions in the nucleus and cytoplasm (**Fig. 1a**). This yielded the number of fluorescent protein molecules within the effective illumination volume. By correlating molecule numbers with fluorescence intensities (**Fig. 1b**), we calibrated the microscope detector and converted protein fluorescence movies to time-resolved protein concentration distribution maps (**Fig. 1c**). Using this automatic experimental pipeline, we acquired a pilot dataset for 28 proteins. Most of these proteins were tagged with EGFP at all alleles of the endogenous locus by targeted recombination using zinc finger nucleases[16] or CRISPR-Cas9 nickase[17], while for some genes stable integration of cDNAs or bacterial artificial chromosomes[18] had to be used (**Supplementary Table S1,** see Online Methods). Time-resolved 3D protein distribution was recorded for 18 dividing cells per protein on average (499 cells in total, ranging from 10 to 35 cells per protein, Fig. 1d), giving us a sufficiently large dataset to develop and test our computational framework.

To assess the accuracy of our quantitative measurements, we compared our data for nucleoporins (NUP107, NUP214, TPR and RANBP2) to expected numbers calculated from the known number of nuclear pores complexes (NPC) per cell and known protein

stoichoimetry in each NPC. The HeLa Kyoto cell line used in this study has about 10,000 NPC in interphase before nuclear envelope breakdown (NEBD)[19]. Assuming a nucleoporin (NUP) stoichiometry as reported [20] (32 NUPs/NPC for NUP107, TPR and RANBP2; 16 NUPs/NPC for NUP214), we can compute the number of NUPs present on the nuclear envelope (NE). Considering a free pool of nucleoporins in the cytoplasm that is included in our measurements, the ratio of our measurements over expected numbers on the NE should be greater than 1. We find that this ratio is on average 1.2 for all four NUPs, underlining the consistency of our measurements with established protein numbers by orthogonal methods.

Definition of mitotic standard time

Traditionally, mitosis is divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase[21]. However, cell division is a continuous process. Except for nuclear envelope breakdown and chromosome segregation that mark the onset of prometaphase and anaphase respectively, the other stages are not separated by sharp kinetic boundaries. Moreover, mitotic progression varies significantly between individual cells (**Fig. 1d** and **Supplementary Fig. S1**). To align the kinetic data from different cells, we therefore first defined a "mitotic standard time" based on objectively measured changes in chromosome morphology. To this end, cell and chromosome boundaries of all imaged mitoses were automatically segmented in 3D using the landmark channels (**Fig. 2a** and **Supplementary Fig. S2**; see Online Methods). Three geometric features were extracted from the segmented data: the distance between the two segregated chromosome masses, the total chromosome volume and the third eigenvalue of the chromosome mass (**Fig. 2b**). Each mitosis movie could thus be represented as a six-dimensional vector sequence of these parameters and their first derivative indicating kinetic transitions. To align the vector sequences, we used a modified Barton-Sternberg algorithm with customized multidimensional dynamic time warping (see Online Methods and **Supplementary Fig. S3a-c**) that locally stretches or compresses the sequences such that sections with similar feature values are assigned to the same standard time point (**Supplementary Fig. S3a**, **b**). After a few iterations, the alignment converged and all vector sequences displayed very similar kinetic signatures (**Fig. 2c** and **Supplementary Fig. S3d**), allowing us to use the final alignment as a mitotic standard time reference (**Supplementary Fig. S4**). We identified reproducible kinetic transitions by the local maxima in the second derivative of the average feature sequences (**Fig. 2d** and

Supplementary Fig. S5, see Online Methods). This approach provides an objective way to discretize the mitotic standard time, which depends on the sampling and the number of cells used. Varying the sampling of our data identified between 19 and 21 stages with a median set of 20 mitotic stages, which we therefore used throughout the study. To check that these mitotic standard stages were biologically relevant we automatically selected the 3D image stack closest to the average feature values of each stage (**Supplementary Fig. S6**). Although the images picked in this way are from different cells, their automatically assigned sequential order reconstitutes a virtual mitosis with an error-free chronology (**Fig. 2e**), in which all classically known mitotic transitions such as nuclear envelope breakdown (between stage 2 and 3), and anaphase onset (between stage 11 and 12), were correctly identified. Moreover, the method could identify previously hard-to-define stages such as the first formation of the metaphase plate in late prometaphase (between stage 7 and 8), and could differentiate between the different anaphase and telophase stages (stage 12 to 17). In addition, the kinetics of chromosome condensation is consistent with previous reports in different cell types [22,23] suggesting that the method could be applied to standardize the mitotic time in other cell types.

A canonical model of the mitotic HeLa cell for interactive exploration of dynamic protein distributions

The mitotic standard time provides an objective way to map all cell images to a standard time reference or bin them to 20 standard stages for averaging. In order to enable visualization, interactive navigation and analysis of all imaged mitotic protein distributions, we next computed the canonical geometry from late prometaphase (stage 7) to cytokinesis (stage 20), where little deviation from rotational symmetry around the division axis occurs. As illustrated with three example cells in late metaphase 1 (stage 10; **Supplementary Fig. S7a**), after translational and rotational registrations based on their chromatin masses (**Supplementary Fig. S7b**), the mean geometry was computed from the cylindrical coordinates of all segmented surfaces of chromatin and cell masses (**Supplementary Fig. S7c-f**; see Online Methods). The canonical geometry model was then reconstructed from the average geometry of several hundred cells for each analyzed mitotic standard stage (**Supplementary Fig. S7g**), preserving the information on cell-to-cell variability (**Supplementary Fig. S7h**).

Evolution of the mitotic standard geometry over the mitotic standard time defines the 4D canonical mitotic cell model (**Supplementary Fig. S7g**), enabling us to

register all cell divisions for the 28 proteins in space and time based on the landmark channels (see Online Methods). For each protein, we mapped each 3D stack to the corresponding mitotic standard stage and then computed the mean and variance of the concentration for each standard voxel and time point of the canonical cell (**Fig. 3a**, see Online Methods). This resulted in 4D concentration maps representing the average behavior and biological variability of each mitotic protein (**Supplementary Fig. S8**). Maps of many proteins can then be freely combined (**Fig. 3b**), to compare their localization patterns, dynamics and abundance and provide intuitive navigation of the integrated data set as illustrated in our web-based interactive atlas (www.mitocheck.org/mitotic_cell_atlas) which allows visualization of multiple user-selected proteins simultaneously at individual mitotic standard stages.

To illustrate the power of integrated data exploration for multiple proteins in the canonical model, we investigated the nuclear/chromosome localization of eight mitotic chromosome structure proteins throughout mitosis (**Fig. 3c** and **Supplementary Fig. S9a-b**). Five out of eight showed a clear chromatin localization from NEBD to anaphase (**Fig. 3c**). Plotting the total number of proteins bound to mitotic chromosomes and in the daughter nuclei against the mitotic standard time provided a quantitative comparison of protein dynamics and revealed (**Fig. 3d-e,** see Online Methods) that the amount of most chromosomal proteins bound to the genome in metaphase is within the same order of magnitude (3 000 – 26 000 per cell and genome), except for TOP2A which shows a 25 times higher abundance, potentially suggesting a structural rather than a purely enzymatic role (**Fig. 3d-e,** see Online Methods; Note: reported numbers represent monomers. Presence as dimers (e.g. TOP2A) would result in a 50% reduced abundance of complexes). We found cohesins to slowly and progressively dissociate from chromatin (RAD21, STAG1, and STAG2), with a final more rapid release of approximately 3 000 remaining cohesin complexes before anaphase onset, indicating that no more than 100 cohesins are sufficient to connect the sister chromatids on an average human chromosome, mostly at the kinetochore (see Online Methods and [24]). Interestingly, chromatin bound cohesins consisted of very similar amounts of the two isoforms containing the HEAT repeat subunits STAG1/2, which summed up to the concentration of the shared kleisin subunit RAD21 (Fig. 3e). This is in contrast to the situation in interphase nuclei, where STAG2-containing complexes dominate[25]. Furthermore, we observed that a significant amount of STAG2 ($p < 0.025$, paired Wilcoxon signed rank-test), but not

RAD21 appears to rebind chromosomes in anaphase, suggesting a potential non-cohesive function on chromosomes at anaphase onset **(Fig. 3d-e, Supplementary Fig. S9a)**. In contrast to the complete dissociation of cohesin, about 17 000 molecules of the chromatin organizer CTCF remain associated with the genome throughout mitosis[26], consistent with a "bookkeeping" mechanism of interphase chromosome architecture. Once chromosome segregation was initiated, KIF4A, TOP2A and CTCF further accumulated on chromatin in anaphase, which suggests a role for these proteins in further DNA compaction that has been observed in anaphase[22] (Fig. 3d-e). Interestingly, the cytoplasmic pool of mitotic chromosome proteins shows a stepwise order of re-entry into the reformed nucleus and different rates of import. CTCF is reimported first with the highest rate (391 proteins/sec), followed by simultaneous import of the cohesin subunits STAG1 and RAD21 (352 and 239 proteins/sec, respectively), while STAG2 and WAPL enter the nucleus later and at a lower rate (69 and 89 proteins/sec, respectively). This shows that mitotic decondensation can proceed in the presence of CTCF and subsequently STAG1-containing cohesin complexes, while WAPL and STAG2 are not present.

In addition to the chromosomal proteins, we also explored the assembly of the machinery that imports them into the nucleus, the nuclear pore complex during anaphase. Consistent with previous observations, cytoplasmic ring components (NUP107, NUP214) assemble early, but surprisingly found that nuclear basket as well as cytoplasmic filaments (TPR and RANBP2) form only much later, at a time when import of CTCF is already completed. This suggests that nuclear and cytoplasmic filaments of the NPC are not required for the rapid import of nuclear proteins needed for re-establishing the interphase genome architecture (**Fig. 3d, Supplementary Fig. S9c**).

Prediction of dynamic co-localization networks of mitotic proteins

To understand which proteins work together where and when inside the cell, it is essential to measure the similarity of their dynamic subcellular distributions. Although the visualization of all mitotic proteins in the canonical mitotic cell allows interactive visual inspection and quantitative exploration of the data (**Fig. 3b**, **Supplementary Fig. S9** and see www.mitocheck.org/mitotic_cell_atlas), visually analyzing similarity becomes difficult for more than three proteins. In order to compute the similarity between all mitotic proteins, we therefore transformed their spatial distribution into numerical features. To this end, we used a segmentation-free approach based on a speeded-up robust features

(SURF) detector[27], to extract a series of so-called interest point clusters from the maximum intensity projection of each 3D mitotic protein image stack (**Fig. 4a** and **Supplementary Fig. S10**; see Online Methods). Each 3D movie was thus transformed into a sequence of 100-dimensional feature vectors, with each feature value representing the fraction of the total amount of the detected cellular protein assigned to the respective interest point cluster (**Fig. 4a**). By averaging the feature vectors of all images of the same protein and mitotic standard stage, the dynamic distribution of all proteins in the dataset could then be represented as a third order tensor of size 28 × 100 × 20 (proteins × features × stages, **Fig. 4b**). Soft clustering with non-negative tensor factorization (NTF) could detect seven clusters of dynamic quantitative protein distribution patterns across all 20 stages (**Fig. 4b**). The identity of the proteins in each pattern revealed a striking correspondence between the statistically defined clusters and major mitotic organelles and structures (e.g. CENPA identifies centromeres/kinetochores, RACGAP1 identifies the midplane in late mitotic stages, **Supplementary Fig. S11**), thus validating our unsupervised approach. Since NTF assigns the fraction of a mitotic protein to each pattern over time (see Online Methods), it reliably deals with promiscuous proteins present in multiple sub-cellular structures. Linking proteins with similar patterns at each time point allowed us to derive a dynamic multigraph (see Online Methods), which showed the dynamic protein co-localization network highlighting the activities of different compartments over time and allowed us to predict where and when proteins may interact (**Fig. 4c**). Results from the above clustering can be used to generate hypotheses that can be visualized in the canonical cell model. Looking for instance at the temporal evolution of AURKB and CDCA8 (aka Borealin), two members of the chromosome passenger complex (CPC), predicts that the two proteins relocate to the midplane (**Fig. 4b**, purple in the right panel) in different proportions and with different kinetics. Since the two proteins are present in a 1:1 ratio in the CPC[28], this observation suggested that a fraction of AURKB is not part of the CPC in the midplane. Exploring the 4D localization of CDCA8 and AURKB in the canonical cell model (**Supplementary Fig. S12**) indeed revealed that while these two proteins partially colocalize at the midbody, AURKB but not CDCA8 shows an additional striking localization in an equatorial cortical ring that contracts as mitosis progresses. This localization of the key mitotic regulator AURKB suggests that it is an integral part of the contractile cytokinetic ring. While unexpected, this observation is consistent with detection of AURKB[29] and its interaction partner INCENP[30] at the lateral edge of the midzone and with its function in cytokinesis[31–33]. This raises the very

interesting possibility that the midplane and cytokinetic ring pools of AURKB have different functions for central spindle architecture and cytokinesis during mitotic exit, which will be very interesting to investigate further in the future.

Automatic measurement of subcellular fluxes predicts the (dis)assembly of mitotic protein machines

Since our clustering of common 4D localization patterns does not a priori yield pure sub-cellular compartments as defined ultrastructurally or by fractionation, we developed a supervised machine learning approach to define subcellular structures using known resident proteins of six compartments/organelles relevant for mitosis: chromosome, nuclear envelope, kinetochore, spindle, centrosome and midbody (see Online Methods). Using the same interest point cluster feature data described above (**Fig. 4a**) as input, we trained a multivariate linear regression model that could assign protein amounts to the six reference compartments (**Fig. 5a**). This allowed us to quantitatively compare the subcellular fluxes between these compartments for all proteins (**Supplementary Fig. S13**).

Mining this data is uniquely powerful to dissect dynamic molecular events inside living cells, including for example the assembly or disassembly of organelles. As an example, we calculated the number of all imaged proteins localized to kinetochores, to investigate the disassembly of this large supramolecular complex essential for cell division. The data allowed us to determine that kinetochore disassembly starts in early metaphase with dissociation of BUB1B and PLK1 followed in late metaphase by BUB1, AURKB, MIS12 and CDCA8 (**Fig. 5b**). In addition, this analysis showed that the stoichiometry of these proteins prior to disassembly differs up to six-fold and that their maximal dissociation rates span over an order of magnitude ranging from 17-173 molecules per second (**Table 1**). The predicted number of ~420 CENPA molecules per kinetochore was consistent with data from biochemical methods[34,35] and the predicted disassembly order was consistent with reports of late dissociation of MIS12[36]. Predictions for the other proteins can be further confirmed by inspection of underlying quantitative live cell images (**Supplementary Fig. S14**), or by exploring the average distribution data of the most interesting proteins in the canonical cell model (www.mitocheck.org/mitotic_cell_atlas).

Our automatic assignment of protein amounts to cellular organelles, thus allowed us for the first time to determine the exact timing, stoichiometry and dissociation rates for multiple mitotic proteins that reside dynamically on kinetochores.

DISCUSSION

With this study we provide an integrated experimental and computational framework to build a comprehensive and quantitative 4D model of the mitotic protein localization network in a dividing human cell. Our model provides a standardized yet dynamic spatio-temporal reference system for the mitotic cell that can be used to integrate quantitative information on any number of protein distributions sampled in thousands of different experiments. Using a pilot data set of 28 proteins of interest, imaged in hundreds of dividing cells, we illustrate the power of this model by mining the data to automatically define dynamic localization patterns to subcellular structures as well as predicting the order, stoichiometry and rates of assembly and disassembly of sub-cellular organelles. Obtaining quantitative information on protein localization in living cells is key to gaining insights into protein dynamics and interactions at temporal resolutions relevant to cellular processes. The information collected here is therefore of high value for building refined dynamic models of mitotic processes. Our computational model allows building an interactive 4D atlas of the human mitotic cell, which we have made available via the MitoCheck web site (http://www.mitocheck.org/mitotic_cell_atlas), to provide the pilot data as a resource and host data on additional mitotic proteins as it becomes available. The digital atlas allows the navigation of freely selectable multiple protein dynamics with a spatial and temporal resolution and continuity that are currently very difficult or impossible to reach with multi-color live cell imaging over the duration of mitosis. We illustrate with mitotic chromosome formation, kinetochore disassembly, NPC assembly and cytokinesis how the knowledge gained through the exploration and mining of the atlas data can be used to formulate new mechanistic hypotheses about the function of proteins inside the cell. The concept of standardizing the spatio-temporal cellular context for analyzing dynamic protein distributions in order to understand cellular processes molecularly as presented here is generic and we envision its adaptation to other essential biological functions such as for example cell migration or cell differentiation.

METHODS

Data and code availability | All images processed in this study are available in the Image Data Resource (http://idr.openmicroscopy.org [37]) under DOI: pending.
Data resulting from processing the images are available on the project web site: http://www.mitocheck.org/mitotic_cell_atlas/downloads. Code used in this study is available from EMBL's git repository at https://git.embl.de/grp-ellenberg/mitotic_cell_atlas. For details see **ONLINE METHODS**.

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AUTHOR CONTRIBUTIONS

B.K, B.N., M.K., R.L., M.M. and N.W constructed and validated GFP knock-in cell lines. A.Z.P. with the help of Y.C and M.W. developed the imaging pipeline. Y.C., A.Z.P., and N.W. performed the calibrated live cell imaging. Y.C., M.J.H., J.K.H., and A.Z.P. developed the computational analysis pipeline. Y.C., J.K.H., M.J.H., A.Z.P., and J.E.

wrote the manuscript. J.M.P. coordinated the Mitosys consortium and supervised part of the work. J.E. supervised the work overall and originally conceived the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1 | Quantitative imaging of mitotic proteins. (a) Automatic calibrated 3D live confocal imaging pipeline. Cells in prophase were identified by online classification, imaged through mitosis in the landmarks and protein of interest channels, and measured by FCS at selected positions. (b) The local protein concentrations determined by FCS fitting linearly correlate with the background subtracted image intensities at the corresponding positions (shown are data acquired on the same day). (c) Example cell showing concentration map resulting from FCS-based intensity calibration (mean Z-projection). (d) Individual cells have different mitotic spatio-temporal dynamics. Scale bar: 10 μm.

Figure 2 | Modeling of mitotic standard time. (a) Cellular and chromosomal volumes were segmented from the landmarks channel. Scale bar: 10 μm. (b) Three morphological features (in red) were extracted from the chromosomal volume. (c) Mitotic standard time was generated in the feature space by multiple sequence alignment visualized here in the feature dimension describing chromosomal volume. (d) Detection of mitotic transitions. Peaks (red circles) above threshold (grey dashed line) were detected on the second derivatives in the feature dimension of chromosomal volume (left). For the second derivatives of all six feature dimensions, a total of 19 peaks defining 19 transitions (grey circles) for 20 mitotic stages were detected (colored). The solid curve shows the maximum projection through the feature dimensions. (e) The standard mitotic cell was represented by the cell closest to the average of each stage. Each mitotic stage was assigned duration (colored line), its duration standard deviation (dashed line) and a biological annotation.

Figure 3 | Visualization of 4D protein distribution maps. (a) Through averaging of a large number of cells, models were generated for all mitotic standard stages with symmetrical geometries. Example image sequences were registered to the standard space of the corresponding mitotic standard stage. A distribution map over time was then generated for each protein by averaging through multiple cells. (b) Average distributions of four proteins are displayed in different mitotic stages. Colored lines indicate mitotic stages according to figure 2e. (c) Maximal intensity projection of the concentration map for chromatin binding proteins. Scale bar:10 μm. (d) Amount of chromatin-bound and nuclear

molecules for eight chromatin remodelers. (e) Fraction of chromatin bound proteins relative to NCAPH2. Shown is the mean and standard deviation. In (d) and (e) TOP2A has been scaled down by a factor 10 for visualization.

Figure 4 | Identification of dynamic protein clusters. (a) SURF interest points were detected (top panel) and assigned to one of 100 clusters of similar interest points. Each image was represented as the distribution of intensity in each of the 100 interest point clusters (bottom panel). Scale bar: 10 μm. (b) Non-negative factorization of the data tensor of 28 proteins × features × mitotic stages (left panel) produced a non-negative tensor of reduced dimension (middle top panel) whose entries can be interpreted as the fraction of protein belonging to each cluster over time (right panel). Scale bar: 10 μm. (c) Dynamic multi-graph of protein co-localization, shown for 5 stages. Each edge colour corresponds to a localization cluster as in (b) and the edge thickness corresponds to the product of the linked genes fractions in the corresponding cluster and can be loosely interpreted as a probability of interactions.

Figure 5 | Analyzing sub-cellular (dis)assembly kinetics using a supervised approach. (a) Example of maximally Z-projected images of marker proteins for the selected subcellular compartments. Scale bar: 10 μm. (b) Kinetics of kinetochore disassembly. The predicted number of molecules localized on kinetochore/centromeres are plotted for eight proteins in the mitotic standard time (left panel) and zoomed in for anaphase (right panel). (c) Model showing the order of protein removal from the kinetochore in anaphase.

TABLES

Table 1 Kinetics of kinetochore disassembly

	Late metaphase	Anaphase kinetochore dissociation	
Gene	Protein number [10^3]	Mitotic standard time [min]	Rate [proteins/s]
BUB1	27.4	31.4	38
AURKB	65.8	33.3	187
CDCA8	32.9	35.0	73
MIS12	7.8	35.4	19

ONLINE METHODS

Cell culture | Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM Glutamine and 1 mM (v/v) Sodium pyruvate at 37 °C and 5% CO_2. Depending on the genetic modification, one or more of the following antibiotics were supplied to the culture at the stated final concentration: Geneticin (Life Technologies) 500 µg/ml, Hygromycin B (Invitrogen) 200 µg/ml or Puromycin (Invitrogen or Calbiochem) 0.5 µg/ml. Once the cells reached 80-90% confluence, they were passaged and only a fraction of the cells were cultured in a fresh dish. All cells tested negative for mycoplasma contamination.

Cell modification | HeLa Kyoto cells were used for genetic modifications and imaging. HeLa Kyoto are hypotriploid with on average 64 chromosomes, thus during mitosis the cells have on average 64*2 = 128 kinetochores[24]. All cell lines used for this project are listed in **Supplementary Table S1** with their providers indicated. Several cell lines were generated within this project as follows: The cell lines expressing PLK1-mEGFP, CEP192-mEGFP and NUP107-mEGFP were generated using the Zinc finger nuclease (ZFN) pipeline as in[38]. Zinc finger nucleases were purchased from Sigma-Aldrich with DNA-binding sequences listed in **Supplementary Table S2**. The other genome-edited cell lines were generated using the CRISPR/Cas9 system[17] with double nicking. For these cell lines, both gRNAs (**Supplementary Table S2**) and the donor plasmid were designed based on ENSEMBL release 75 and transfected together into HeLa Kyoto cells with jetPrime (Polyplus) according to the manufacturer's instruction. A single clone was selected using our previously developed validation pipeline[38,39]. For 4 out of 20 genome-edited cell lines (BUB1B-EGFP, TPR-mEGFP, mEGFP-NUP107 and CEP192-mEGFP) we detected in the Western-Blots (anti-GFP) a band of the size of free GFP. Therefore, for these cell lines, the total free amount may be overestimated. In order to label the chromosomal volume, an H2B-mCherry[5] cDNA was transfected into some genome-edited cell lines with Fugene6 (Promega) according to the manufacturer's instructions. The pmEGFP2-N1-NES construct was generated by sub-cloning two tandem repeats of mEGFP (mEGFP2)[40] and the NES of MAPKK (NLVDLQKKLEELELDEQQ) into the pEGFP-N1 vector (Clontech Laboratories). The pmEGFP2-N1-NES construct was transfected into HeLa Kyoto cells and cells with stable expression were selected by culturing with the appropriate

antibiotics. Single cells or a cell population with the desired expression level were harvested for imaging by fluorescence activated cell sorting (FACS, performed by the EMBL Heidelberg facility).

Calibrated fluorescence confocal microscopy | Confocal microscopy was performed on a Zeiss LSM780, Confocor 3, laser scanning microscopes using 40x, NA 1.2 water DIC Plan-Apochromat objectives and the GaAsP detectors equipped with an incubation chamber (EMBL workshops). Cells were imaged at 37 °C in a CO_2-independent medium (Life Technologies) fluorescently colored with 500 kDa Dextran (Life Technologies) - DY481XL (Dyomics) produced in house. Time-lapse imaging was performed using the ZEN 2012 software as well as in-house software applications. The acquisition was supported by an in-house developed objective cup and a water pump, such that water drops were regularly supplied to the objective-sample interface. Before starting imaging, a number of positions were selected manually. During live cell imaging, the microscope determined the focus automatically by performing line-scan imaging of the reflection signal of the 633 nm laser. The vertical position of the glass bottom was determined as the position with the maximum reflection intensity, and used as reference for acquiring a volume of the specimen at a particular depth.

HeLa Kyoto cell lines with H2B-mCherry were imaged live using an excitation laser at 561 nm every 5 min for about 16 hours on the Zeiss 780 microscopy system. Three confocal planes were acquired at a resolution of 0.32 µm in x and y and 2.5 µm in z. Images were projected in z by taking the maximum intensity value. Images of the H2B signal were analyzed on the fly by the Micronaut software (Gerlich lab, IMBA, Vienna) using a support vector machine classifier that was trained beforehand (with the software CellCognition, http://www.cellcognition.org/) to distinguish between cells in interphase, prophase, mitosis (prometaphase till telophase) and artefacts (apoptosis, on the border of the imaging field, out of focus, too low expression). The classification score for the prophase, interpreted as the probability of a cell being in the class of interest, was output, and a pre-defined threshold was used to make a decision on whether imaging setups for mitotic cell acquisition should be activated. According to how different the sample's H2B-mCherry expression levels were from the training set, the threshold on class probability was set between 0.85 and 0.96. Once a prophase cell was found, it was then imaged using a different imaging setup. For our purpose, mitotic cells were imaged live every 90 seconds for 31 z-planes with a spatial resolution of 0.25 µm in x-y and 0.75 µm in z with a

488 nm laser (high expression of H2B-mCherry allowed it to be excited at 488 nm and produce adequate signal). For cells that do not express H2B-mCherry we used SiR-DNA to stain the chromatin (Spirochrome, final dilution 50 nM added 2 hours before imaging). Cells were imaged with the 633nm laser (3 confocal planes every 7.5 min at the same resolution as for H2B-mCherry) and processed as for H2B-mCherry.

The signal from the GaASP detector was separated into three channels: GFP, varied from 490 to 552 nm depending on the expression level to avoid detection saturation; mCherry, 587 – 621 nm (**Supplementary Fig. S2a**), and Dy-481XL, 622 – 695 nm (**Supplementary Fig. S2b**). For SiR-DNA we used 622 – 695nm. Once the mitosis was recorded for 40 frames, a single-plane image was then acquired at 2.5 μm above the cover glass surface. Using an in-house-developed Fiji (http://fiji.sc/Fiji) macro, the image was thresholded using the method developed by[41] and the object closest to the image center with a proper size was selected as one of the two daughter nuclei in the cell of interest. The segmented nuclear boundary was fitted with an ellipse. FCS measurements were performed with the 488 nm laser and the APD detector at 505 – 540 nm at two positions in and four around the nucleus with a distance of 2 μm to the ellipse boundary for 30 seconds each.

A manual quality control was then performed. Videos of cells with no expression of the protein of interest, with wrongly selected FCS positions (e.g. outside of the cell) or without anaphase onset were excluded from further processing.

Segmentation of the landmarks | A fully automated computational pipeline was implemented in MATLAB (MATLAB R2017a, The MathWorks, Inc., Natick, Massachusetts, United States) to segment and track cells of interest and reconstruct chromosomal and cellular surfaces. The pipeline was composed of three major steps: segmentation of chromosomal volume, segmentation of cell volume and extraction of parameters out of the landmarks geometry. Chromosomal regions were segmented from the mCherry channel which had high H2B–mCherry signal and very low Dextran-Dy481XL intensity (**Supplementary Fig. S2a**) or from the SiR-DNA channel which had no crosstalk from other channels. In order to perform isotropic 3D image processing, adjacent x-y planes were linearly interpolated along the z direction. A 3D Gaussian filter was applied to reduce the effects of noise. To detect chromosomal regions, the filtered image stack was binarized first using a multi-level thresholding method as described in[23]. In this approach, a global Otsu threshold[42] was determined for the entire stack and the threshold was then

adapted for each 2D slice, validated by the connectivity of binary components in 3D. Tiny connected components were removed from the binary image leaving only chromosomal components from all cells in the imaging field. All components were used as seeds for the detection of the cell boundary in a later stage. The connected chromosomal volume in the x-y center of the first frame identified the cell of interest due to the centering step in the imaging pipeline. The cell of interest was tracked sequentially through the entire image sequence using a nearest neighbor approach. At each time point, an event of chromosome segregation was also probed by analyzing the chromosomal volume around the tracked location. Once segregation was detected, both daughter nuclei were tracked in the subsequent frames (**Supplementary Fig. S2c**).

The cell region was segmented from the Dy481XL channel showing the cell-free regions in high intensities and histone signal with low intensities (**Supplementary Fig. S2b**). Upon interpolation and filtering as for the chromosomal segmentation, a ratio image was created by dividing the filtered image stack of the mCherry channel by that of the Dy481XL channel in order to diminish bleed-through signal from the H2B-mCherry channel. The ratio image stack was then binarized as described above. When using SiR-DNA there is no bleed-through in the Dy481XL channel when excited at 488. In this case the Dy488XL was directly binarized. To separate individual cell regions, the nuclear seeds previously detected were used, considering the fact that each cell region can have only one or two chromosomal volumes. This was implemented by applying a marker-controlled watershed algorithm[43]. To obtain a better separation between touching cells, the algorithm was applied on the distance transformed image that made use of the geometric properties of the cell surface. The cell region of interest was defined by taking the connected region(s) containing the detected chromosomal volume(s) of interest (**Supplementary Fig. S2d**).

The chromosomal mass at each time point was represented by its three orthogonal eigenvectors and associated eigenvalues where the eigenvector with the largest eigenvalue represented the longest elongated axis of the chromosomal volume. Metaphase frames were automatically detected based on the low value of the smallest eigenvalue of the chromosomal volume. Division axis for metaphase cell was then predicted by taking the eigenvector having the minimum eigenvalue. By definition, this vector is always orthogonal to the metaphase plate. Using the predicted axis in the first and last metaphase frame, axes for the remaining frames were propagated backwards and forwards for stages before and after metaphase, respectively, where the eigenvector

with the smallest discrepancy in angle to the axis predicted for the adjacent frame was used (**Supplementary Fig. S2e**). For further analysis, the plane orthogonal to the division axis going through the centroid of both daughter nuclei was predicted as the midplane. Segmented landmarks were 3D reconstructed and visualized for quality control. Cells having few time points over or under segmented were reprocessed with different parameters or using the results of adjacent time points correctly segmented as constraint.

Image processing and calibration | Image processing and calibration were performed according to[44]. Before each calibrated live cell confocal microscopy experiment, the focal volume was calibrated using a 20 nM solution of Alexa488 (Life Technologies), and single mEGFP brightness was calibrated by performing FCS measurements on the HeLa Kyoto expressing mEGFP[38]. All FCS measurements were processed using Fluctuation Analyzer[6]. Autocorrelation functions of dye solutions were fitted using a one-component diffusion model with triplet-like blinking, and measurements of fluorophore-fused proteins were fitted using a two-component anomalous diffusion model with fluorescent protein-like blinking. The effective confocal volume was calculated from

$$V_{eff} = (4 \cdot \pi \cdot D \cdot \tau)^{\frac{3}{2}} \cdot \kappa$$

where D was the diffusion coefficient of the Alexa488 which is 464 $\mu m^2/s$ at 37 °C and κ the structural parameter. The averaged time passing through the confocal volume τ and the structural parameter (typically between 4-7) was fitted to the ACF of Alexa488. The number of fluorescent molecules within a confocal volume was calculated by multiplying the fitted number of molecules (N) with correction factors for background and photobleaching[6]. As proteins might exist in complexes with multiple molecules, a count per molecule (CPM) value was used to correct the number of molecules. As reference, the CPM value of mEGFP was used as measured in the HeLa cells expressing mEGFP where the mean value of all mEGFP measurements were taken. If the CPM of a measurement of a fusion POI within a cell was larger than that of the mEGFP, the fitted number of molecules was corrected by multiplication with the ratio between the two. Finally, the local concentration of the measured protein was determined as the corrected number of molecules divided by the effective confocal volume. As quality control of the FCS measurements, we pre-defined thresholds and deleted data points with too low coefficient of variation R^2 or APD counts or too high fitting χ^2 or bleaching or outlier CPM values.

The calibration of the image acquired with the GaAsP detector was based on the assumption of linear correlation between the local protein concentration and the EGFP intensity which we could verify (**Fig. 1b**). The averaged intensity of the GFP channel in cell-free areas was considered as background. For all measurement points the coefficient ρ between local protein concentration and background-corrected imaging intensity, mean filtered with a 9 × 9 pixels window to avoid noise, was calculated by performing a linear regression. The 3D protein concentration map was generated by multiplying the pixels intensities with the linear coefficient ρ. The protein number in each voxel was obtained by multiplying the concentration with the voxel volume. The absolute protein abundance could be calculated by summing up the map over the cell volume. After NEBD and before the nuclear envelope reforms, we estimated the number of proteins bound to chromatin by subtracting the cytoplasmic average concentration (representing the background concentration of proteins that freely diffuse between the cytoplasmic and chromosome volume) from the average protein concentration on the chromosome mask (**Fig. 3d-e** and **Supplementary Fig. S9**). Finally, to obtain the number of proteins, the concentration difference is multiplied by the number of voxels of the chromosome mask and the voxel volume.

Modeling of the mitotic standard time | The mitotic standard time was modeled in a six-dimensional feature space using three morphological features of the chromosomal volume: the distance between the two daughter nuclei, the total volume and the third eigenvalue (**Fig. 2b**) and their first derivatives. The model was generated by aligning 132 mitotic image sequences using the Barton-Sternberg multiple sequence alignment algorithm (**Supplementary Fig. S3c**)[45]. The two sequences with the smallest distance to the average of all sequences were selected to initiate the alignment and each of the remaining sequences was then aligned to the average among all aligned sequences. The alignment was implemented as a modified multidimensional dynamic time warping[46] where the total Euclidean distance over time between the pair of sequences was used as the objective of the optimization (**Supplementary Fig. S3a**). The timeline of the averaged sequences was calculated as the mean of the alignment matrix as shown in **Supplementary Figure S3b**. The Barton-Sternberg algorithm was terminated after four rounds as the standard deviation over time remained stable after 2 rounds (**Supplementary Fig. S3d**). The mitotic standard time was defined at a temporal resolution of 15 seconds by subsampling the averaged timeline. In order to find

transitions in the mitotic standard time, the second derivative of the model at each time point for each feature dimension was calculated from

$$x_t'' = |(x_{t-} - x_t) - (x_t - x_{t+})|,$$

$$\text{where } x_{t-} = \sum_{i=t-18}^{t-1} x_i/18, \quad x_{t+} = \sum_{i=t+1}^{t+18} x_i/18.$$

Peaks above a pre-defined threshold were selected across all dimensions as transitions. In the later part of the model where the values of the second derivatives were generally low, small peaks were selected as additional transitions such that no stage between two transitions lasted longer than 12 minutes. Furthermore, transitions with lower values were deleted to ensure a minimum duration of 1.5 minutes for each stage (**Supplementary Fig. S5**).

Modeling of the canonical cell | To support spatial averaging, all cells assigned to the same standard mitotic stage were registered into a common reference coordinate system to give them the same location and orientation. To this end, a virtual coordinate system was defined with its origin at the center of a volume chosen large enough to accommodate all cells after registration. Landmarks (i.e. the cell boundary and chromosomal volumes) were then registered to the virtual coordinate system by applying a transformation function involving translation and rotation in 3D. This transformation function was estimated such that the predicted cell axis was aligned with the x axis in the virtual coordinate system. This transformation was applied to both landmarks to preserve their interrelationship in the registered image stacks as shown in **Supplementary Fig. S7a** and **b**. Bicubic interpolation was used when applying the transformation[47].

Registered landmarks were subsequently represented using a cylindrical coordinate system that transforms 3D coordinates into radial distances providing greater flexibility in shape analysis (**Supplementary Fig. S7c**). To this end, we converted landmarks in each plane along the z axis and along the predicted cell axis to polar coordinates in which object boundaries are represented by their radial distances from the object centroid (**Supplementary Fig. S7c, d**). As the centroids were aligned on the z axis, the cylindrical representation was formed by concatenating into a vector the polar representations for all planes (**Supplementary Fig. S7d**). After chromosome segregation, two separate cylindrical representations were used to encode each of the two daughter nuclei. In this case, the cylindrical axis of each chromosome passes through the centroid of that chromosomal volume.

The standard mitotic space represented by the averaged landmarks was computed in three steps. In the first step, the cylindrical coordinate vectors were averaged separately for each landmark across all cells within each standard mitotic stage (**Supplementary Fig. S7e**). The average vectors were then transformed back to a Cartesian coordinate system from which binary image stacks were generated. In a second step, to reconstruct the landmarks, the average volume of each landmark was obtained by combining two binary image stacks: one obtained using the z axis as the cylinder axis and the other using the cell axis as cylinder axis (**Supplementary Fig. S7f**). This combination involved first taking the intersection between the two binary images and then extending it until the average volume of all the cells belonging to the mitotic stage being processed was reached (**Supplementary Fig. S7f**). Because multiple frames of a cell could be assigned to the same mitotic standard stage, cells could have unequal contributions to each stage with some cells represented more than others at a given stage. To ensure uniform contribution from each cell towards the average mitotic space, in the third step, for each given mitotic stage and for each cell, the frame that was most similar to the average shape obtained in step 2 was selected. These selected cells were then used to re-compute the average shape of the corresponding mitotic stage as described above. This final average shape was also used to calculate the standard deviation of all cells in the same mitotic stage. Average mitotic space and standard deviation were generated for all the stages (7-20) for each of the landmarks (examples in **Supplementary Fig. S7g** and **h**).

Generation of the protein density map | Standard mitotic spaces were used as reference to register and integrate protein distributions from many different cells to generate protein density maps (**Fig. 3a**). All calibrated protein concentration maps having the same protein in a given mitotic stage were registered first to the corresponding standard mitotic space using the predicted cell division axis. This transformed all individual protein image stacks to the same coordinate system. Bicubic interpolation[47] was used during the rotation. Registered image stacks were then accumulated in the standard mitotic space. Pixels outside the segmented cell region and mapped outside the standard mitotic space were discarded. A protein density map was then created by averaging the accumulated intensities in the standard mitotic space (**Fig. 3a**). Density maps of all proteins for mitotic stages 7-20 were estimated in the same way and can be explored on http://www.mitocheck.org/mitotic_cell_atlas.

Feature extraction of images | The protein z-stack concentration map was processed using a Gaussian filter (Matlab smoth3 function with a kernel of size [3 3 1] and standard deviation 0.65) followed by a maximum projection along the z-axis and normalization to the theoretical saturation intensity. SURF interest points[27] were then detected on the image resized to a 0.063 μm resolution using three octaves each including four Haar wavelet filters at different sizes from 9-by-9 till 99-by-99 pixels ranging from about half a micrometer to more than six micrometers. Interest points were further selected such that most of the protein signals were counted in one of the interest points. Each of these interest points was then described by a numerical vector quantifying features in the following four categories: locations relative to the landmarks (four features), correlation to the H2B signal or the predicted midplane/midbody volume according to the localization of the interest point (one feature), flattened soft spin image features[48] describing the intensity distribution within an interest point (30 features), and summarized uniform Local Binary Patterns (uLBP)[49] describing the orientation of the signal (4 features).

5% of the cells were randomly selected and their interest points were used to construct a training set for identifying clusters of interest points with similar features (**Supplementary Fig. S10**). All training interest points were first separated into 16 clusters by their localization feature and the ¼-level of their metric values. Interest points in clusters with a sufficient size were then further clustered based on the correlation features separated by pre-defined thresholds followed by a dbscan[50] clustering for each sub-cluster in the reduced feature space covering 85% of the variance according to a principal component analysis[51] on the uLBP and spin image features[48]. The final clustering step was performed only for the clusters with the highest contrast value in their location category based on the spin image features where the interest points were separated into homogeneous bright, structured bright and dim clusters by pre-defined threshold. The total number of clusters was not deterministic since the training set was randomly generated but eight rounds of clustering yielded between 87 and 100 clusters and a run with 100 clusters was used for further analysis. Interest points in the same cluster share similar textures (**Fig. 4a**). All interest points in the remaining images in the data set were then each assigned to one cluster. The total intensity within each interest point was then counted and the fraction of intensities recorded in each interest point cluster was calculated for each cell so that each image was represented by a 100-dimensional feature vector with a sum of one.

Non-negative tensor factorization | Each protein was represented at each mitotic stage by the average of all its vectors present at that stage. Due to binning of consecutive imaging time points, a cell can be represented by several vectors at a given mitotic stage. These duplicates were replaced by their average resulting in each cell being represented by only one vector per mitotic stage. The resulting dataset is a three-dimensional tensor $\underline{\mathbf{X}}$ of 28 proteins × 100 features × 20 mitotic stages. We view canonical subcellular localizations as latent features of the data, that is, we assume that, at any time point, the observed vector for a protein was generated by a combination of the different canonical subcellular localizations the protein occupied at this stage. A protein vector x can then be expressed as the product of a subcellular localization membership vector z and a matrix $\mathbf{A}$ of canonical subcellular localization features: $x = z\mathbf{A}$. Therefore, we wish to model our data tensor $\underline{\mathbf{X}}$ such that for each frontal (temporal) slice $\mathbf{X}_t$,

$$X_t = Z_t A + E_t (t = 1,2, \dots ,20)$$

where $\mathbf{Z}_t$ is a matrix whose rows are localization membership vectors and $\mathbf{E}_t$ is a matrix containing the errors.

Given that all feature values are non-negative, a possible solution for each time point can be found by non-negative matrix factorization (NMF) of individual matrices $\mathbf{X}_t$[52]. However, processing time points independently results in loss of information with the undesirable effect that different canonical localizations are learned for different time points. Simultaneous non-negative factorization of a set of matrices is a special form of non-negative tensor factorization (NTF) which can be reduced to a standard NMF using column-wise unfolding of the data tensor $\underline{\mathbf{X}}$[53]:

$$X = ZA + E$$

where $\mathbf{X}$ is formed by vertically stacking the $\mathbf{X}_t$ matrices and $\mathbf{Z}$ is formed by the correspondingly stacked $\mathbf{Z}_t$ matrices and $\mathbf{E}$ contains the errors. $\mathbf{Z}$ and $\mathbf{A}$ are then found using multiplicative updates[52] to minimize the objective function $||\mathbf{X} - \mathbf{ZA}||$ where $||.||$ indicates the Frobenius norm. As a final step, the rows of $\mathbf{Z}$ are normalized to sum one. Values in $\mathbf{Z}$ can be interpreted as fractions of the amount of protein (captured by the features) present at each canonical localization.

The method requires choosing the number k of canonical subcellular localizations we want to represent our data with. There is no good strategy for finding this number a priori because increasing k corresponds to a higher resolution of the localization

description e.g. a low *k* results in lumping all chromatin proteins together while a higher *k* resolves kinetochore proteins from other chromatin proteins. Thus the optimal number of subcellular localizations is partly subjective, depending on the level of granularity desired. However, we can use heuristics to help guide the choice of *k*. If the number of selected canonical localizations is too low, many proteins will share the same temporal profile, i.e. their corresponding vectors in $\mathbf{Z}_t$ will be highly similar for all time points. As more canonical localizations are added, we can expect more proteins to resolve into distinct profiles, i.e. the similarity between their corresponding vectors will decrease until eventually adding more canonical localizations will not improve resolution and similarity will stop decreasing. Similarity between vectors across time points can be measured using Tucker's congruence coefficient (TCC)[54]. Therefore, for each value of *k* from 2 to 25, we plot the fraction of TCC values above 0.6. The value of *k* for which the fraction of highly similar proteins reaches a low value plateau indicates that there are enough canonical localizations to describe each protein individually and therefore this value of *k* represents an upper bound on the number of canonical localizations. Following this procedure, *k* was set to seven for the current data. Because the NMF algorithm can converge to a local minimum of the objective function, ten runs with random initialization of the matrices were performed and the run with lowest value of the objective function was kept. A flattened representation of the resulting tensor can be obtained by assigning a different color to each cluster and plotting each protein distribution as a bar chart in which the height of each color band at each time point is proportional to the fraction of the protein amount in the corresponding cluster (**Supplementary Fig. S11**). A dynamic multigraph can be derived from the cluster memberships as follows: First an edge type is defined for each cluster. If two genes share a cluster at a given time point, then an edge of that type is added between them at that time point. The edge weight is set to the product of the linked genes fractions in the corresponding cluster and can be loosely interpreted as a probability of interaction. For visualization, only edges with a weight greater than an arbitrary threshold (here set to 0.3) were kept (**Fig. 4c**).

Analysis of proteins localization kinetics using supervised annotation | A multivariate linear regression model with a multivariate Gaussian response was trained with an elastic net regularization and non-negativity constraints on the coefficients with the feature vectors described above as predictors and localization vectors as response. The response vectors were defined using cells with tagged proteins known to be specific

markers of unique subcellular compartments (**Supplementary Table S3**) as follows: For each of the marker proteins, the fraction of total intensity in the foreground was determined by Otsu thresholding of the 3D image stack and the corresponding protein amount assigned to the compartment with the complement assigned to cytoplasm. Each cell is thus represented by a 7-dimensional response vector containing the fraction of the tagged protein in the following compartments: chromatin, kinetochore, centrosome, spindle, midbody, nuclear envelope and cytoplasm. To deal with the compositional nature of this data, all features and response vectors are transformed using the additive log-ratio transformation[55] with the inverse hyperbolic sine function as a generalized logarithm to handle occurrences of 0. The model with the best fit using 5-fold cross-validation was selected.

The predictions from the model were transformed back to proportions using the inverse of the log-ratio transformation then multiplied by the total number of proteins to predict the absolute number of molecules in each mitotic subcellular structure for each image. Predictions were then smoothed by local polynomial regression fitting.

To compute the anaphase dissociation kinetics for each kinetochore protein (**Table 1**), we fitted each curve between 30 min and 42 min mitotic standard time (late metaphase to telophase) with a 4 parameter sigmoidal decay function:

$$y = \frac{a - d}{(1 + bc^{(-time)})} + d$$

whose first and second derivatives were analytically calculated. The time of disassembly was defined as the point at which the second derivative is equal to 0 (inflection point of the curve). The disassembly rate was computed as the minimum value of the first derivative in the time interval.

Data and code availability | Full accessibility to data and code is available as follows:

- All original images (including segmentation mask for both cellular and chromosomal volume) and protein concentration maps are available in the public Image Data Repository (IDR) (http://idr.openmicroscopy.org; [55]). DOI: pending.
The images are also available for download on the mitotic cell atlas web site http://www.mitocheck.org/mitotic_cell_atlas/downloads/v1.0/mitotic_cell_atlas_v1.0_fulldata.zip (~0.5 TB).

- All source code is accessible on EMBL's GitLab instance: https://git.embl.de/grp-ellenberg/mitotic_cell_atlas and can be downloaded or cloned using the command
git clone git@git.embl.de/grp-ellenberg/mitotic_cell_atlas.git

A version of the source code is also available for download on the project web site at http://www.mitocheck.org/mitotic_cell_atlas/downloads/v1.0/mitotic_cell_atlas_v1.0_src.zip. Instructions to run the code are provided as a README file together with the source code. An example data set to run and test the source code can be downloaded from http://www.mitocheck.org/mitotic_cell_atlas/downloads/v1.0/mitotic_cell_atlas_v1.0_exampledata.zip.

- The data supporting the spatiotemporal mitotic cell model and the analysis is publicly available from the mitotic cell atlas website (http://www.mitocheck.org/mitotic_cell_atlas/downloads/v1.0) and contains:

- Segmentation masks for the landmarks (i.e. cell boundary and chromosome mass(es)) as TIFF files (directory *mitotic_cell_model/binary_masks*).
- Snapshots of the 3D rendering of each of the spatial models in VRML and TIFF formats (directory *mitotic_cell_model/snapshots*).
- Two movies (orthogonal and oblique views) created from 3D reconstructed average landmarks (cell boundary and chromosome mass(es), directory *mitotic_cell_model/movies*).
- Average concentrations of each protein at individual mitotic stages as mat files, TIFF stacks, and tab-delimited text files (directory *protein_distributions*).
- Feature data used for the analysis (to produce figures 4, 5, S11 and S13) in a tab-delimited text file (file *cell_features.txt*). This file can be used directly as input to the notebooks available in the code repository. This file also contains the mitotic standard time and stage assigned to each cell image.
- Canonical localization data (file *canonical_mitotic_clusters.h5*).
- Dynamic graph (file *dynamic_graph_adjacency_matrices.h5*).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 | Eleven cells expressing AURKB-EGFP showing different mitotic spatio-temporal dynamics indicated by the different timing of prometaphase 1, late metaphase 1, late anaphase and cytokinesis 1 aligned at prophase. Scale bar: 10 μm.

Figure S2 | Overview of the segmentation and prediction of the division axis (a) - (b) Raw image stacks. Single x-y plane image in mCherry (587 – 621 nm, a) and DY481XL (622 – 695 nm, b) detection channels. (c) Detected chromatin markers where boundaries of the chromosomal volume of interest are marked in red. (d) Output of watershed transform on ratio image where boundary of the detected cell of interest is marked in green. (e) Reconstruction of cell and chromosomal surfaces in 3D (gray) and the predicted division axis (red). Scale bar: 10 μm.

Figure S3 | Algorithm for generating the mitotic standard time model. (a) Scheme showing the principle of dynamic time warping implemented here for achieving the alignment of a pair of time-resolved sequences shown in (b) as an example. (c) Modified Barton-Sternberg algorithm to align 132 sequences. (d) The cumulative standard deviation over time remained nearly constant after the 2nd round indicating that at termination (4th round) a stable alignment was achieved.

Figure S4 | All features used for generating the mitotic standard time model before and after alignment. Grey line: normalized feature value over time of a single cell. Black line: the average of all sequences.

Figure S5 | Objective detection of major mitotic transitions of the mitotic standard time. Peaks in the second derivatives (red circles) above a pre-defined threshold (grey lines) were detected in all feature dimensions (upper six panels, normalized) as mitotic transitions. Additional smaller peaks (blue circles) were detected to ensure a maximum duration of 12 minutes for each standard stage and transitions were deleted (grey circles) such that all stages had a minimal duration of 1.5 minutes.

Figure S6 | Selection of the representative cell for each mitotic standard stage. All images in the mitotic standard time training set assigned to mitotic standard stage no. 10 were projected from their six-dimensional feature space to a two-dimensional space using multidimensional scaling. The image with the shortest distance (red rectangle) to the averaged feature value through the entire stage (red dot) across all six dimensions was selected as the representative cell.

Figure S7 | Generation of standard mitotic stage by combining two cylindrical representations. Examples of cells in mitotic stage no. 10 (a) were registered using the predicted cell division axis as shown in (b). (c) Transformation between Cartesian and cylindrical coordinate systems. (d) Example cellular and chromosomal surfaces (grey) were transformed into the cylindrical coordinate system using two cylindrical axes (z-axis or predicted division axis) marked in yellow. (e) Average cellular and chromosomal surfaces in cylindrical coordinate systems. (f) Union (U) and intersection (∩) of the averaged landmarks volumes represented in the Cartesian coordinate system that were then combined to generate final cellular and chromosomal surfaces shown in the first image in (g). By averaging of a large number of cells, models were generated for all mitotic standard stages with symmetrical geometries and example stages 10, 14, 16 and 19 are shown in (g). (h) The spatial variation of the mitotic standard spaces shown in (g).

Figure S8 | Single x-y plane showing the concentration map (a) and its standard deviations (b) of KIF11 in standard mitotic spaces from stage 7 to 20. Scale bar: 10 μm.

Figure S9 | Chromatin remodelers and NUPs localization. (a-b) Maximal intensity projection from the mitotic standard model for three chromatin remodelers (WAPL, STAG1, and STAG2) and (b) four nucleoporins at selected standard mitotic stages. Scale bar: 10 μm. (d) Nucleoporin localization as function of mitotic standard time. The curves for STAG2 and WAPL are shown as a reference and are identical to the data from Fig. 3d.

Figure S10 | The pipeline of definition of interest point clusters using a subset of the data. 936 images (corresponding to 5 % of the entire data set) were randomly selected from the data set constructing a pool of interest points. Each interest point was numerically described with a 40 dimensional feature vector encoding the intensity distribution, localization and contrasts to the interest point neighborhood. Combining k-d-tree-like and

thresholding-based clustering with dbscan[32], the interest points were grouped into 100 clusters where interest points in the same cluster share similar textures. The boundaries identified during the clustering were then used for the annotation of the remaining interest points of the data set (**Fig. 4a**).

Figure S11 | Quantitative evolution of protein subcellular localizations inferred from non-negative tensor factorization of the proteins × features × time tensor. Each subcellular localization cluster was assigned a different color and named using known information on proteins belonging to that cluster. The height of each color band at each time point is proportional to the fraction of the protein amount in the corresponding cluster at that time point. Genes were grouped by complete linkage clustering followed by optimal leaf ordering.

Figure S12 | Concentration maps of chromosome passenger complex proteins AURKB and CDCA8 in anaphase. (a) AURKB concentrates in an outer ring and a central disk. Most of CDCA8 remains on chromatin and after AURKB has already relocalized, between late anaphase and early telophase, only a small CDCA8 fraction colocalizes with AURKB in the central disk. (b) Color displaying CDCA8 was adapted to make it's localization in the central disk visible.

Figure S13 | Prediction of protein molecule numbers on major mitotic subcellular structures using the supervised annotations. The color scheme is adjusted to the most similar cluster identified using NTF (**Supplementary Fig. S11**). Cytoplasm values are divided by 10.

Figure S14 | Maximum concentration Z-projection of example cells expressing different kinetochore proteins over mitosis. Images with very similar mitotic standard time were selected for each stage. The time points correspond to mitotic standard stages 3, 7, 11, 12, 13, 14, 15, and 20 respectively. Scale bar: 10 μm.

SUPPLEMENTARY TABLES

Supplementary Table S1: List of cell lines

Gene name	Tagging method	Cell line	Source	Cells analyzed after quality control
KIF11	BAC	HK cDNA H2B-mCherry BAC mKIF11-GFP #2354	A. Hyman, MPI-CBG Dresden, Germany [56]	15
MIS12	BAC	HK BAC mMIS12-LAP cDNA H2B-mCherry cDNA #2341	A. Hyman, MPI-CBG Dresden, Germany [57]	35
TUBB4B	BAC	HK cDNA H2B-mCherry BAC mTUBB4B-LAP #2637	A. Hyman, MPI-CBG Dresden, Germany [57]	28
RACGAP1	BAC	HK cDNA H2B-mCherry BAC LAP-mRACGAP1 #2362	A. Hyman, MPI-CBG Dresden, Germany [57]	19
CDCA8	BAC	HK cDNA H2B-mCherry BAC mCDCA8-LAP #2607	A. Hyman, MPI-CBG Dresden, Germany [57]	13
NEDD1	BAC	HK cDNA H2B-mCherry BAC mNEDD1-LAP #311	A. Hyman, MPI-CBG Dresden, Germany [57]	28
CENPA	cDNA	HK cDNA EGFP-CENPA cDNA H2B-mCherry pool	T. Hirota, Cancer Institute Tokyo, Japan [58]	21
NES	cDNA	HK cDNA H2B-mCherry cDNA NES-mEGFP2 pool	J. Ellenberg/EMBL, this work	17

PLK1	ZFN	HK ZFN PLK1-mEGFP #24 cDNA H2B-mCherry pool	J. Ellenberg/EMBL, this work	16
AURKB	ZFN	HK ZFN AURKB-mEGFP #H24 cDNA H2B-mCherry pool	J. Ellenberg/EMBL [38]	14
BUB1	CRISPR	HK CRISPR mEGFP-BUB1 #63 cDNA H2B-mCherry pool	J. Ellenberg/EMBL, this work	12
NUP107	ZFN	HK 2xZFN mEGFP-NUP107 #26, 31	J. Ellenberg/EMBL [19]	16
RANBP2	CRISPR	HK CRISPR mEGFP-NUP358/RANBP2 #97	J. Ellenberg/EMBL [25]	22
NUP214	CRISPR	HK CRISPR mEGFP-NUP214 #2-12	J. Ellenberg/EMBL, this work	14
TPR	CRISPR	HK CRISPR TPR-mEGFP #171	J. Ellenberg/EMBL, this work	15
CEP192	ZFN	HK ZFN CEP192-mEGFP #15	J. Ellenberg/EMBL, this work	13
CEP250	CRISPR	HK CRISPR CEP250-mEGFP #1A-142	J. Ellenberg/EMBL, this work	19
NCAPH2	CRISPR	HK CRISPR mEGFP-NCAPH2 #1	J. Ellenberg/EMBL, this work	20
TOP2A	CRISPR	HK CRISPR mEGFP-TOP2A #102	J. Ellenberg/EMBL, this work	16
KIF4A	CRISPR	HK CRISPR mEGFP-KIF4A #173	J. Ellenberg/EMBL, this work	21

WAPL	CRISPR	HK CRISPR WAPL-EGFP	J.M. Peters/IMP [59]	12
STAG1	CRISPR	HK CRISPR STAG1-EGFP #H8	J.M. Peters/IMP, this work	22
STAG2	CRISPR	HK CRISPR STAG2-EGFP #F2	J.M. Peters/IMP, this work	13
RAD21	CRISPR	HK CRISPR RAD21/SCC1-EGFP	J.M. Peters/IMP [60]	18
CTCF	CRISPR	HK CRISPR CTCF-EGFP #F2	J.M. Peters/IMP, this work	16
BUB1B	CRISPR	HK CRISPR EGFP-BUB1B #M04-A03	J.M. Peters/IMP, this work	20
ANAPC2	CRISPR	HK CRISPR mEGFP-ANAPC2 #M21-P1-A11	J.M. Peters/IMP, this work	14
MAD2L1	CRISPR	HK CRISPR MAD2L1-EGFP #M11-B11	J.M. Peters/IMP, this work	10

The LAP tag has EGFP as fluorescent protein

Supplementary Table S2: Recognition sequences and guide RNAs

Gene	Genome-editing tool	Sequences
AURKB	ZFN	CGCCTGATGGTCCCTgtcattCACTCGGGTGCGTGTGTT
PLK1	ZFN	TCGGCCAGCAACCGTCTCaaggccTCCTAATAGCTGCCC
CEP192	ZFN	CTTGTCATTCAAACAGATGaaggcaAGAGTATTGCTATTCG
NUP107	ZFN	TCAGTACTGATGgtggcaGCTGAGCCCGAAGTC
BUB1	CRISPR/Cas9D10A	ACCAGACGGACACTTACTGA GGGCGCCTGGGGTTCGGGCC
RANBP2	CRISPR/Cas9D10A	GGCGCGTGAGACCAGCGCTC GAGGCGCAGCAAGGCTGACG
NUP214	CRISPR/Cas9D10A	GCAGCCAACGCTGCCTCCCA CGGCGCGATGGGAGACGAGA
TPR	CRISPR/Cas9D10A	CTCCTCTCCCTCCCATTGCA CAGAGGAAATATTAATTAAA
CEP250	CRISPR/Cas9D10A	CTGCTACCTGGAGGCGGCTT ACAGACAGAAGACTGTGTCA
MAD2L1	CRISPR/Cas9D10A	GTCATCCTCAGTCATTGAC TAATTGTAATTTTGAAATG
BUB1B	CRISPR/Cas9D10A	CGCCGCCATCCTGCATTCC GGTGCTCTGAGGTAGGTAC
ANAPC2	CRISPR/Cas9D10A	CGCAGCCATGACGCGCACA CGCCGCGCCGAGCGAATCT
RAD21	CRISPR/Cas9D10A	CACCGCTTATATAATATGGAACCT CACCGCAAATTGCCCCCATGTGTA
STAG1	CRISPR/Cas9D10A	CACCGTCTTCAGACTTCAGAACAT CACCGGTTTCTCATCATTTTTCTA
STAG2	CRISPR/Cas9D10A	CACCGCACAGATTTAATTGTGTAC CACCGCTCTCTCTCATTAGGTTCT
CTCF	CRISPR/Cas9D10A	CACCGCAGCATGATGGACCGGTGA CACCGGAGGATCATCTCGGGCGTG
WAPL	CRISPR/Cas9D10A	CACCGCTAAGGGTAGTCCGTTTGT CACCGTGGGGAGAGACCACATTTA
NCAPH2	CRISPR/Cas9D10A	ACCGCAGGGCTGCCTTCCGA CCGTTCCCTCCCGGACATGG

ZFN cut sites are indicated in lower case. For CRISPR/Cas9 editing, the first sequence is the antisense gRNA binding site, the second one is the sense gRNA binding site (double nicking approach).

Supplementary Table S3: Reference structures for supervised model

Localization	Gene	Mitotic standard stages
Nuclear envelope	NUP107	15-20
Chromatin	NCAPH2	1-20
Kinetochores	CENPA	1-20
Centrosomes	CEP192	1-20
Spindle	TUBB4B	4-20
Midbody	RACGAP1	12-20

Figure 1

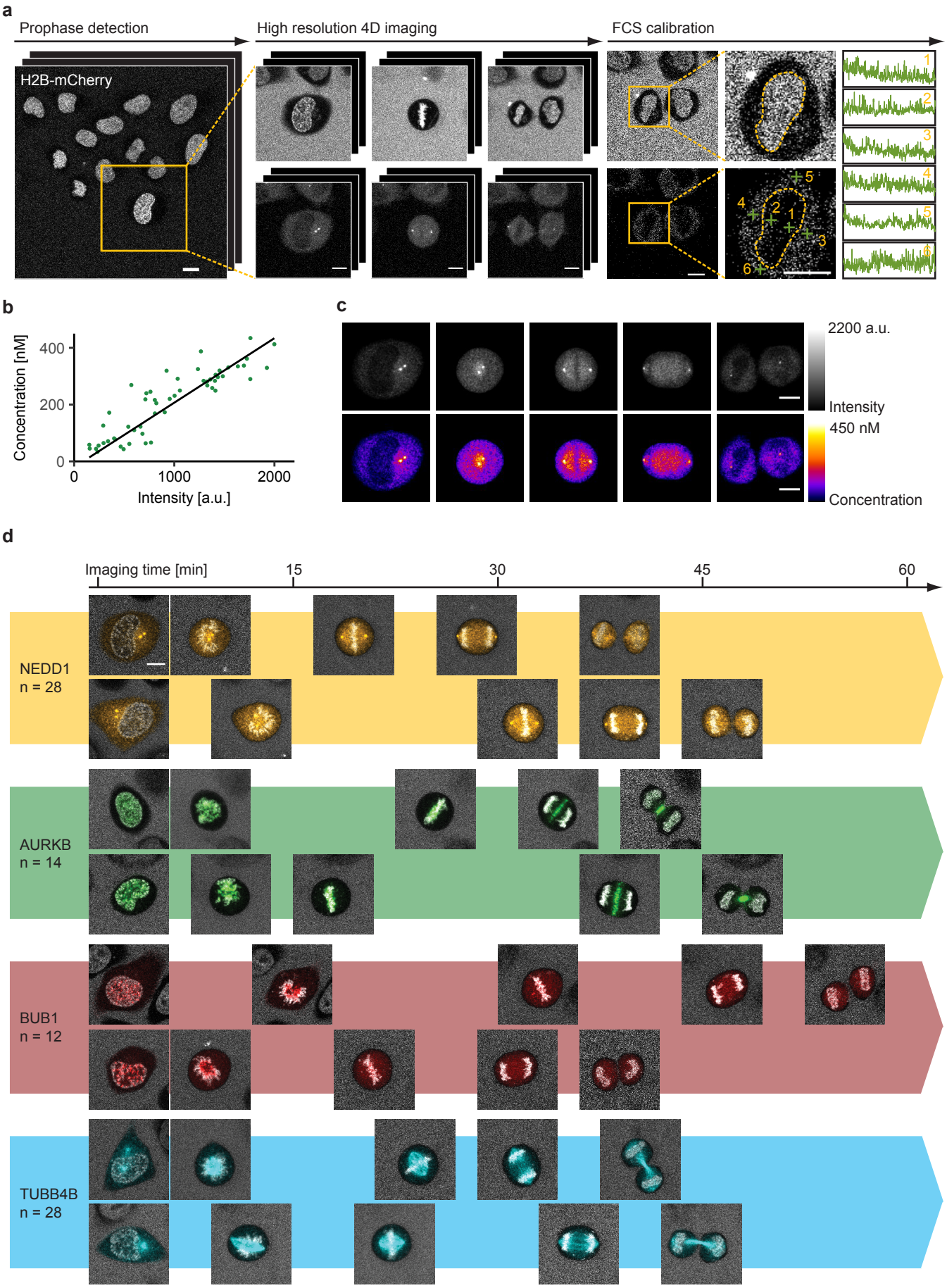

a
Prophase detection
High resolution 4D imaging
FCS calibration
H2B-mCherry
b
Concentration [nM]
400
200
0
0
1000
2000
Intensity [a.u.]
c
2200 a.u.
Intensity
450 nM
Concentration
d
Imaging time [min]
15
30
45
60
NEDD1
n = 28
AURKB
n = 14
BUB1
n = 12
TUBB4B
n = 28

Figure 2

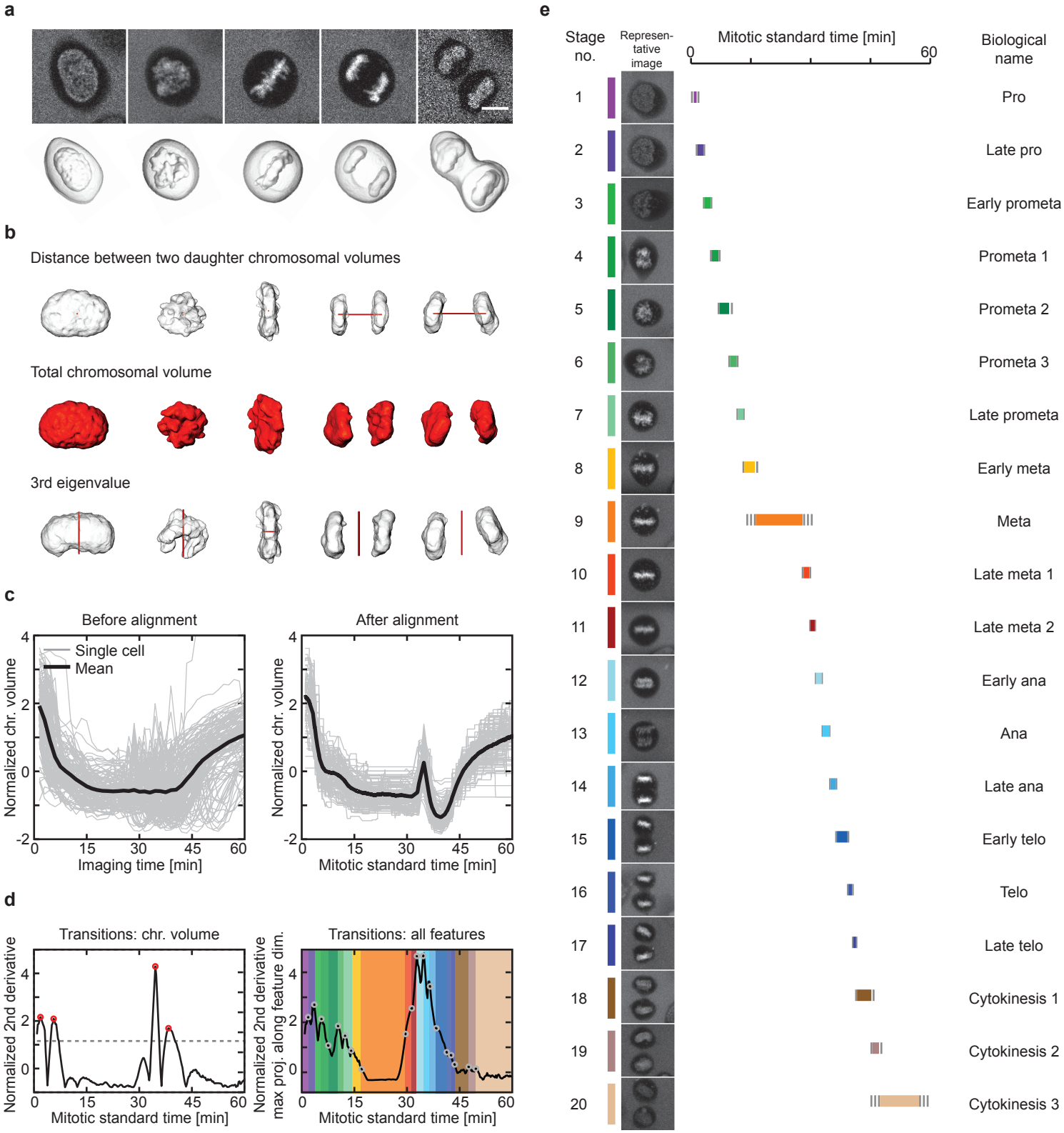

a
b
Distance between two daughter chromosomal volumes
Total chromosomal volume
3rd eigenvalue
c
Before alignment
After alignment
Single cell
Mean
Normalized chr. volume
Imaging time [min]
Mitotic standard time [min]
d
Transitions: chr. volume
Transitions: all features
Normalized 2nd derivative
Normalized 2nd derivative max proj. along feature dim.
Mitotic standard time [min]
e
Stage no.
Represen-tative image
Mitotic standard time [min]
Biological name
1 Pro
2 Late pro
3 Early prometa
4 Prometa 1
5 Prometa 2
6 Prometa 3
7 Late prometa
8 Early meta
9 Meta
10 Late meta 1
11 Late meta 2
12 Early ana
13 Ana
14 Late ana
15 Early telo
16 Telo
17 Late telo
18 Cytokinesis 1
19 Cytokinesis 2
20 Cytokinesis 3

Figure 3

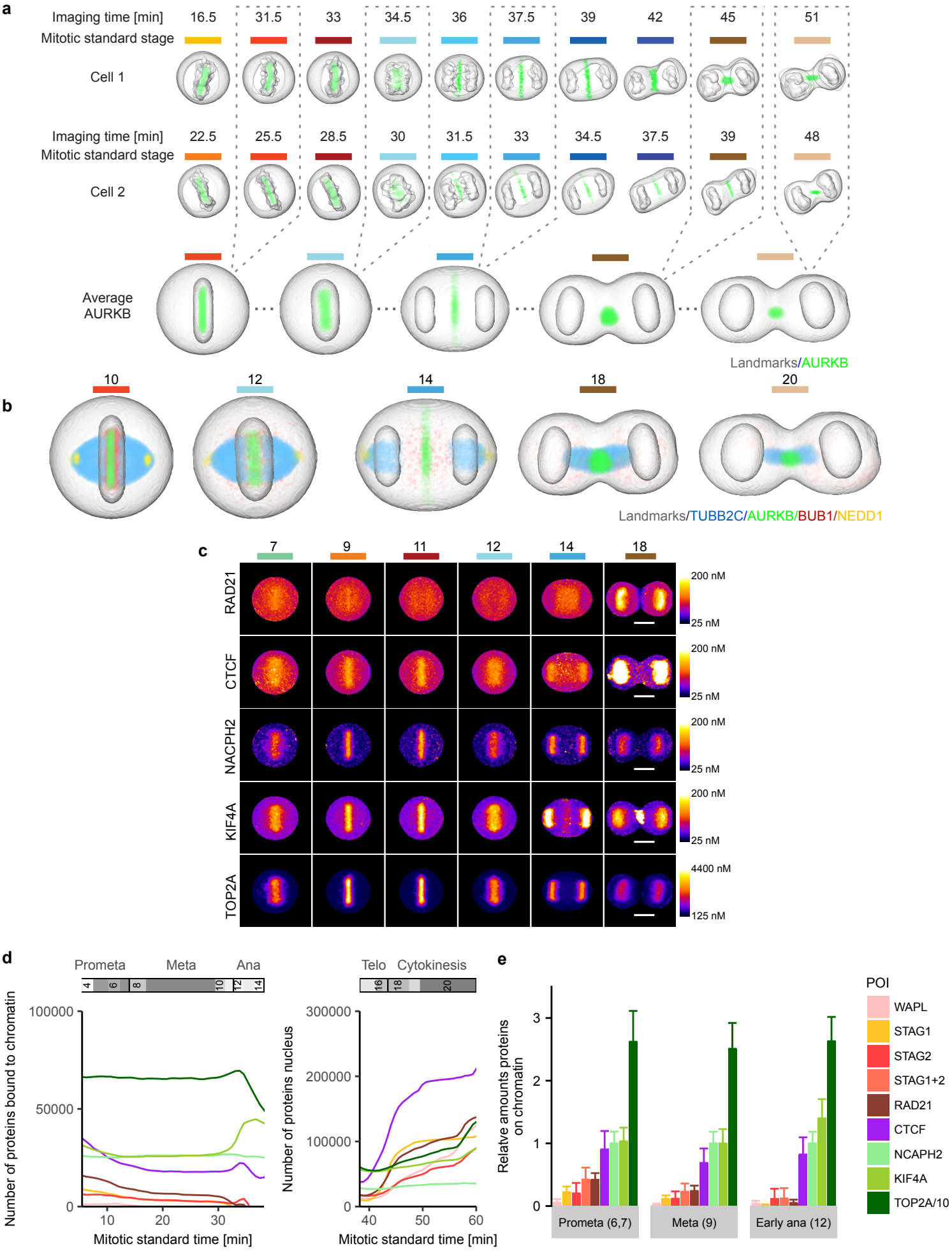

a
Imaging time [min]
16.5
31.5
33
34.5
36
37.5
39
42
45
51
Mitotic standard stage
Cell 1
Imaging time [min]
22.5
25.5
28.5
30
31.5
33
34.5
37.5
39
48
Mitotic standard stage
Cell 2
Average AURKB
Landmarks/AURKB
b
10
12
14
18
20
Landmarks/TUBB2C/AURKB/BUB1/NEDD1
c
7
9
11
12
14
18
RAD21
CTCF
NACPH2
KIF4A
TOP2A
200 nM
25 nM
4400 nM
125 nM
d
Prometa
Meta
Ana
Telo
Cytokinesis
Number of proteins bound to chromatin
Number of proteins nucleus
Mitotic standard time [min]
e
Relatve amounts proteins on chromatin
Prometa (6,7)
Meta (9)
Early ana (12)
POI
WAPL
STAG1
STAG2
STAG1+2
RAD21
CTCF
NCAPH2
KIF4A
TOP2A/10

Figure 4

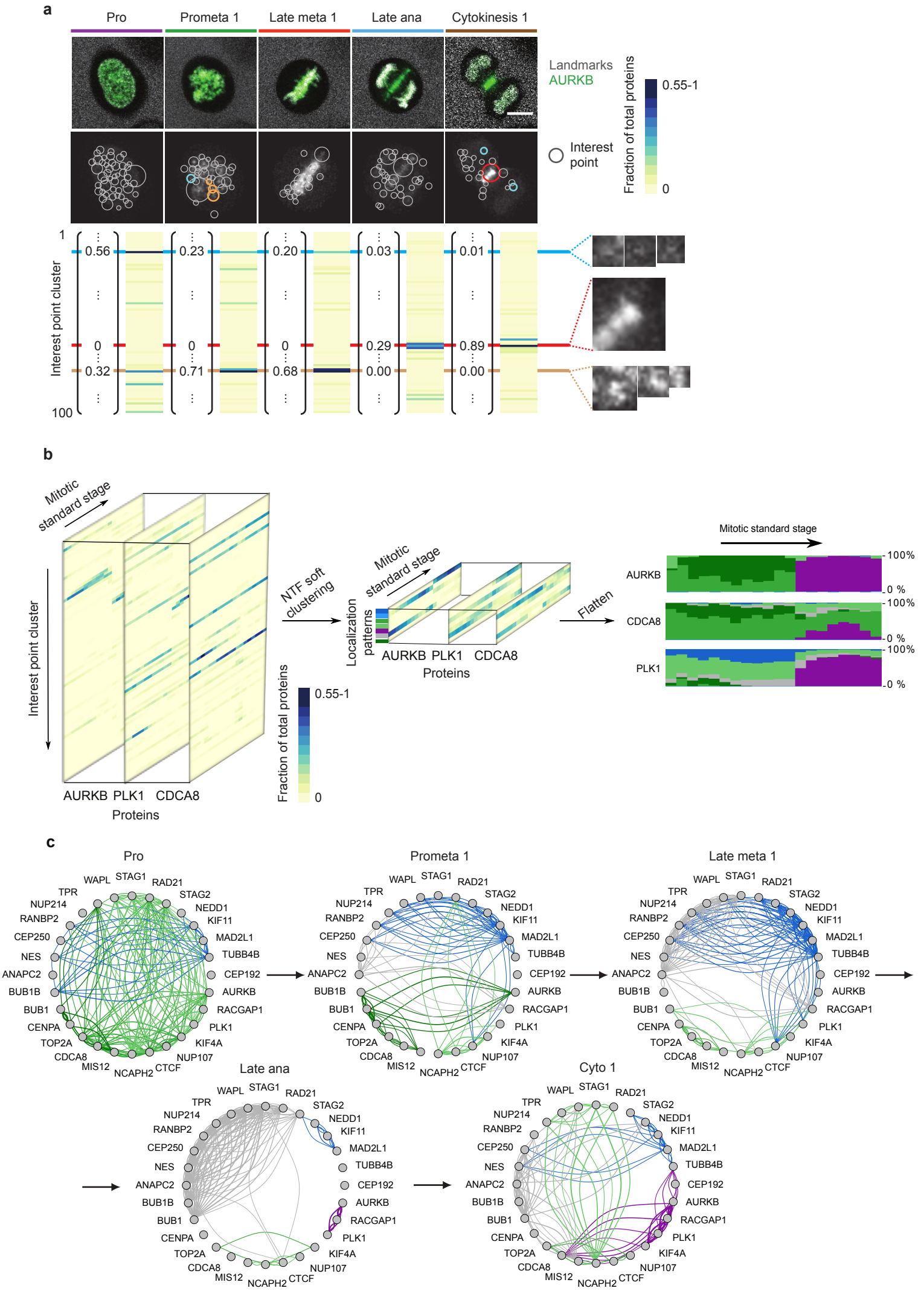
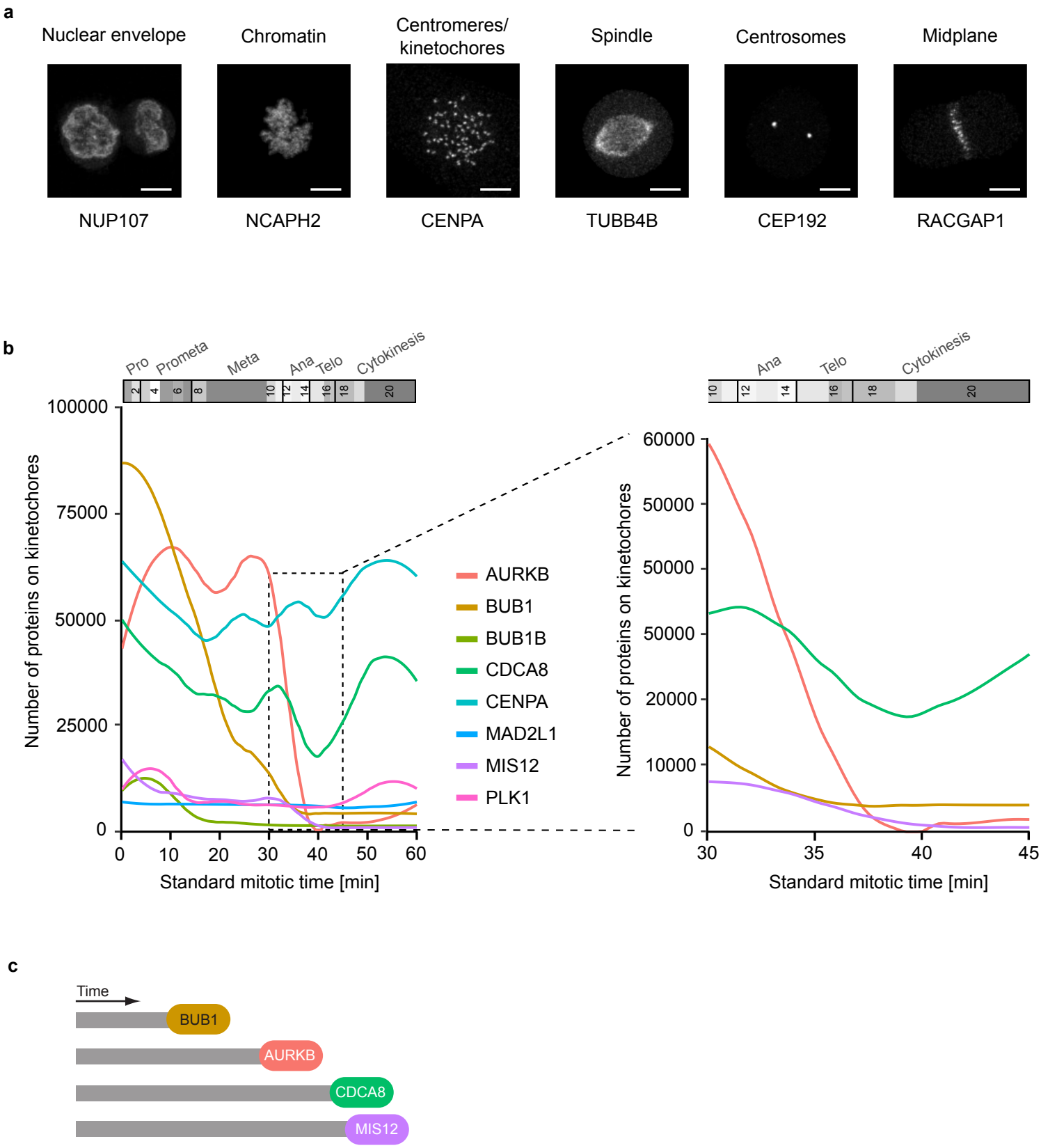

a
Pro
Prometa 1
Late meta 1
Late ana
Cytokinesis 1
Landmarks
AURKB
Interest point
Fraction of total proteins
0.55-1
0
Interest point cluster
1
100
0.56
0.23
0.20
0.03
0.01
0
0.29
0.89
0.32
0.71
0.68
0.00
0.00
b
Mitotic standard stage
Interest point cluster
AURKB PLK1 CDCA8
Proteins
NTF soft clustering
Localization patterns
Flatten
Mitotic standard stage
AURKB
CDCA8
PLK1
100%
0 %
c
Pro
Prometa 1
Late meta 1
Late ana
Cyto 1
WAPL STAG1 RAD21 STAG2 NEDD1 KIF11 MAD2L1 TUBB4B CEP192 AURKB RACGAP1 PLK1 KIF4A NUP107 CTCF NCAPH2 MIS12 CDCA8 TOP2A CENPA BUB1 BUB1B ANAPC2 NES CEP250 RANBP2 NUP214 TPR

Figure 5


a
Nuclear envelope
Chromatin
Centromeres/ kinetochores
Spindle
Centrosomes
Midplane
NUP107
NCAPH2
CENPA
TUBB4B
CEP192
RACGAP1
b
Pro
Prometa
Meta
Ana
Telo
Cytokinesis
Number of proteins on kinetochores
Standard mitotic time [min]
AURKB
BUB1
BUB1B
CDCA8
CENPA
MAD2L1
MIS12
PLK1
c
Time
BUB1
AURKB
CDCA8
MIS12