**PART B**

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# Part B-1

## 1. Excellence #@REL-EVA-RE@#

### *1.1 Quality and pertinence of the project's research and innovation objectives (and the extent to which they are ambitious, and go beyond the state of the art)*

#### *1.1.1 Summary and State-of-the-art.*

Sustaining accurate segregation of genetic material is essential to life. In eukaryotes, centromeric DNA is responsible for nucleating the assembly of the megadalton kinetochore complex, which connects the chromosome to spindle microtubules and enables DNA segregation to two daughter cells[1]. The budding yeast *Saccharomyces cerevisiae* is a crucial model system for centromere and kinetochore studies, as its kinetochores, the smallest known, represent the organizational module that gives rise, by multiple repetitions, to larger kinetochores such as those of humans. Although progress has been made in understanding the organization of the inner kinetochore Ctf19$^{CCAN}$ complex, a crucial knowledge gap remains regarding how the inner kinetochore is assembled on native centromeric DNA. *CENStruK* aims to bridge this gap by reconstituting the key step in kinetochore assembly, harnessing required protein chaperones, and applying biophysical and structural studies of the endogenous *CEN*-kinetochore complex using cryo-EM and cryo-ET. My studies will not only shed light on a fundamental unknown in chromosome biology but will also inform further work in other eukaryotes.

#### *1.1.2 Overview of the action.*

The native 120 bp centromere (*CEN*) of yeast is sufficient to determine the site of kinetochore assembly *in vivo*[2]. *CEN* sequence specificity is provided by three elements: CDEI, an ~8 bp sequence, to which the trans regulator Cbf1c binds, CDEII, a ~70-170 bp sequence (depending on the lineage), to which Ndc10 and the centromeric histone H3 variant Cse4 bind, and CDEIII, a ~26 bp imperfect palindrome, to which the Cbf3 core complex (Cbf3c, consisting of Skp1, Ctf13, and Cep3) binds[3–5]. The "CDE binding proteins" serve as the base upon which the inner kinetochore, consisting of the Ctf19$^{CCAN}$ complex, is assembled. However, yeast's native *CEN* DNA does not bind to centromeric histone octamers *in vitro*[6,7]. As a result, contemporary structural methods rely on reconstituting the inner-kinetochore complex on non-native, engineered centromeric DNA[6,8]. It is not immediately obvious whether these structural models accurately depict the endogenous state, as the engineered centromeric DNAs have no centromere activity *in vivo* and fail to recruit key kinetochore proteins *in vitro*[6,7]. Thus, contemporary understanding of the mechanisms that structurally guide the assembly of *CEN* DNAs with the inner kinetochore remain incomplete.

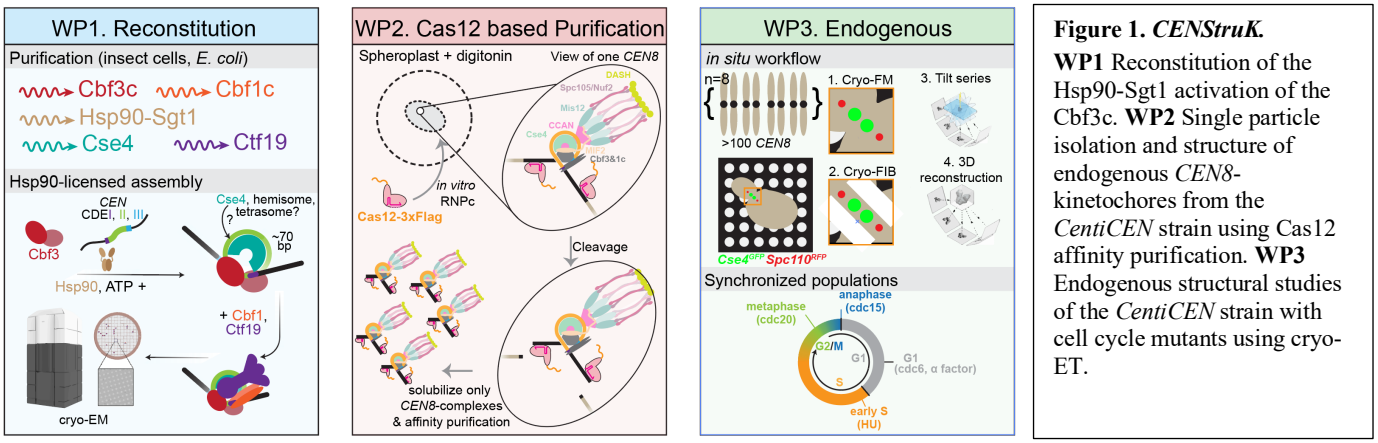



**Figure 1. *CENStruK.***
**WP1** Reconstitution of the Hsp90-Sgt1 activation of the Cbf3c. **WP2** Single particle isolation and structure of endogenous *CEN8*-kinetochores from the *CentiCEN* strain using Cas12 affinity purification. **WP3** Endogenous structural studies of the *CentiCEN* strain with cell cycle mutants using cryo-ET.

The assembly of the yeast inner kinetochore is initiated by the binding of the Cbf3 complex to *CEN* DNA[7]. This key event is licensed by the chaperone Hsp90 and its co-chaperone Sgt1[9,10], via an interaction with Skp1[11]. However, this step has not yet been reconstituted *in vitro*. Additionally, evidence suggests that yeast *CEN* DNA is wrapped by a non-canonical nucleosome containing the histone H3 variant Cse4[12–15]. Thus, in WP1 **I will attempt to reconstitute the key Hsp90-Sgt1-dependent assembly of the Cbf3c on *CEN* DNA *in vitro***. Next, using diverse nucleosome templates (e.g., octa–, tetra–, and hexa-metric), along with reconstituted Ctf19$^{CCAN}$ complex, I will assess the stability and binding of these reconstituted objects. Finally, I will determine the complete structure of the yeast inner *CEN*-kinetochore complex following activation of Cbf3c by Hsp90 using single-particle cryo-EM

List of Acronyms used in *CENStruK*.

***cryo-EM/ET**: cryogenic electron microscopy/electron tomography, **CEN**: yeast centromere, **CCAN**: constitutive centromere-associated network, **WP**: work package, **PQC** Protein Quality Center, **MPI**: Max Planck Institute, **MPG** Max Planck Society, **HPC**: High-performance computing, **MCB**: Mechanistic Cell Biology.*

Studying endogenous structures does not require the reconstitution of cell cycle-dependent regulation of the *CEN*-kinetochore complex. However, native yeast kinetochores are inherently heterogenous due to the different native centromeric DNAs of each chromosome[16]. Moreover, the number of *CEN*-kinetochores per cell is inherently low, with only 16 per haploid cell. Overcoming these pitfalls could open up an avenue for structural studies of the endogenous yeast *CEN*-kinetochore complex. *CENStruK* will develop a novel approach that unlocks this opportunity. In WP2 **I will develop and deploy the *CentiCEN* strain**, which will then be used in WP3 for **single-particle cryo-EM and *in situ* structural studies**. With homogenized *CEN* DNA and after rapid polyploidization, each *CentiCEN* cell can house more than 100 identical *CEN*-kinetochore complexes. Using this unique strain for structural studies, I will purify endogenous *CEN*-kinetochore particles via an innovative designer Cas12 affinity purification method. In parallel to single-particle experiments, I will also use the novel strain to investigate the centromere kinetochore structure *in situ* using cryo-ET. The central goal is to achieve a mechanistic understanding of *CEN* DNA function. To do this, I will use cell cycle mutants and blocking chemicals to study the native *CEN*-kinetochore structure across the cell cycle, using both cryo-EM of single particles and *in situ* studies with cryo-ET. In this way, we can use time-tested tools of yeast genetics to reliably obtain highly-pure samples ideal for cryo-EM and cryo-ET.

### *1.1.3 Originality and innovative aspects of the planned research.*

Numerous studies have focused on the structure of yeast and mammalian kinetochores, and the overall architecture of the Ctf9$^{CCAN}$ is well established[6,17–19]. However, none have satisfactorily provided a unified structural rational for the nearly ubiquitous importance of *CEN* DNA and the centromeric histone Cse4$^{CENP-A}$. Paramount to CENStruK is the Hsp90-liscensing step of Cbf3c binding to DNA, however to-date this step has only be validated with whole-cell lysates[9], thus our *in vitro* reconstitution will shed light on how Hsp90 alters Cbf3c binding to *CEN* DNA. Thus, CENStruK with address these key molecular connections and steps. Furthermore, current methods for purifying whole kinetochores use harsh cell lysis methods, and combined with intrinsic sample heterogeneity, this has limited the effectiveness of endogenous structural studies[20]. The methodology of *CENStruK* therefore relies on gentle cell lysis and solubilization of kinetochores. Additionally, because the purification is Cas12-based, no kinetochore protein need be tagged, preserving its native structure. The flexibility of the *CentiCEN* strain also allows us to simultaneously pursue *in situ* structure determination by cryo-ET. By increasing the number of *CEN*-kinetochores per cell, we can reduce the total number of cells that need to be milled for data acquisition. Finally, the tools and technology developed for *CEN*-kinetochores purification will be useful for structural studies of other endogenous chromatin bound structures.

## *1.2 Soundness of the proposed methodology (including interdisciplinary approaches, consideration of the gender dimension and other diversity aspects if relevant for the research project, and the quality of open science practices).*

### *1.2.1 Important Challenges.*

#### 1.2.1.1 Selection of yeast as a model system for kinetochore structure.

*In vitro* structural studies of the kinetochore have been difficult due to the complex's large size and flexibility[21–23]. In recent years, *in situ* approaches have become popular for resolving the atomic structures of large molecular complexes[24]. However, no successful reports of atomic models for the *CEN*-kinetochore complex have been published. It can be argued that the focus on mammalian systems[21,22], where each centromere is defined by multiple (~20) kinetochores attached to several spindle microtubules, may thwart such efforts due to the heterogeneity, flexibility, and redundancy of these connections[23]. The uniqueness of yeast's single centromeric nucleosome offers an intriguing advantage as each kinetochore must be fully assembled and bound to both a single centromere and microtubule to function. This is why, after addressing the aforementioned challenges of the yeast system, we have selected it as the model to study endogenous kinetochore structure.

#### 1.2.1.2 What makes centromeric chromatin?

The prevailing model of centromeric chromatin proposes that an octamer containing the variant histone Cse4$^{CENP-A}$ wraps centromeric DNA in a canonical left-handed superhelix.[25] Intriguingly, this arrangement necessitates the unwrapping of >30 bp of DNA at each end of the nucleosome[6]. As a result, *in vitro* octameric Cse4$^{CENP-A}$ nucleosomes can only be solubilized by addition of a stabilizing single-chain antibody fragment.[6,8] Additionally, it has so far proved impossible to reconstitute the Cse4$^{CENP-A}$ octamer on native *CEN* DNA. Consequently, structural studies use engineered sequences that do not support centromere function. This naturally raises the question of whether the Cse4$^{CENP-A}$ octamer is the actual substrate upon which the yeast kinetochore is assembled *in vivo.* Multiple topological studies of yeast centromeres *in vivo* have shown that they drive the formation of positively supercoiled DNA,

dependent on the binding of the inner kinetochore complex, Cbf3c.[13,14] One possible explanation is that *CEN* DNA is wrapped in a right-handed manner, which is not easily compatible with an octameric histone complex. In contrast, sub-octameric complexes (hemisome or tetrasome) have the flexibility to accommodate a right-handed path of DNA[14], potentially driven by the binding of the Cbf3c. Remarkably, the Cbf3c induces a bend of ~55˚ to *CEN* DNA upon its binding and this geometry accounts for the observed of positive supercoiling.[14,26] In this configuration a loop of approximately ~70 bp (the average length of CDEII) would be formed, into which a sub-octameric complex could be accommodated. Inspired by these observations, we will reconstitute both hemisome and tetrasome substrates for assembly into Hsp90-assited Cbf3c-*CEN* complexes.

### *1.2.2 Integration of methods and disciplines.*

To achieve the proposed work packages (**WP**), *CENStruK* uses an interdisciplinary approach that integrates biochemistry, yeast genetics, and electron microscopy. To deliver on **WP1**, I will purify all necessary components required for determining the structure of the yeast inner kinetochore. Next, the Cbf3c licensing step by Hsp90 will be conducted in tandem with binding assays to *CEN3* DNA, by gel shift and pull-down methods. Once a suitable *CEN*-Cbf3c complex is formed, I will additionally bind the Ctf19$^{CCAN}$ complex using similar methods. I will then gain the expertise in cryo-EM sample prep and data acquisition in order to determine the structure of Hsp90-licensed yeast inner kinetochore. To deliver on **WP2** and **WP3**, I will transfer my knowledge of yeast genetics and strain engineering to the host Institution by developing the *CentiCEN* strain. This strain will then be used in parallel experiments: one using a novel Cas12-based purification method for single particle cryo-EM and the other an *in situ* approach using cryo-ET, together with the goal to determine the endogenous *CEN*-kinetochore structure.

**WP1. Reconstitution of the yeast inner kinetochore**

WP1.A: Expression and purification of the yeast inner kinetochore. Since host laboratory previously reconstituted the yeast Hsp90-Sgt1 and various histone complements, the focus will now be on producing only the components of the yeast inner kinetochore. Each subcomplex will be expressed in multi-vector plasmids in either insect of bacterial cells. I will use established protocols and methods based on the host Lab's expertise in reconstituting the human inner kinetochore. Quality control of expressed proteins will be carried out using the Protein Quality Center (PQC) at the MPI-Dortmund. The PQC offers standardized analysis of purified proteins such as mass photometry, mass spectrometry, and SEC-MALS. This will ensure all subunits are present in expected molar ratios. WP1.B: Reconstitution of the Hsp90-liscening of the active *CEN*-Cbf3 complex. I will use both solution and solid phase biochemical assays to determine if reconstituted Hsp90-Sgt1 (e.g. with or without ATP) is sufficient to activate Cbf3c binding to native *CEN* DNA. Protein binding to *CEN* DNA will be monitored by gel shift assays and also pulldowns using a biotinylated *CEN* DNA. Next, I will reconstitute the *CEN*-Cbf3 complex with different types of Cse4$^{CENP-A}$ containing nucleosomes (i.e., octasome, hemisome, tetrasome), both with and without the Cse4 chaperone, Scm3. Sub-octameric nucleosome will be reconstituted following established protocols.[27] I will also utilize a Aurora B kinase phosphomimic mutation in the Cse4 END domain to enhance Scm3 binding to Cse4, which is reported to improve Cse4 loading onto *CEN* DNA.[28] Finally, I will test the binding of the Ctf19$^{CCAN}$ complex to the *CEN*-Cbf3-Cse4 complex, and this integrated structure will be analyzed using cryo-EM.

**WP2. Construction of the *CentiCEN* yeast strain and Cas12-based kinetochore purification.**

WP2.A: Polyploid *CentiCEN* strain construction. An optimal yeast strain for endogenous kinetochore structural studies would have homogeneous centromeres that are in sufficient quantity. I will use the *CEN8*x16 strain that has 15/16 of its centromeres precisely replaced with a 340 bp fragment of *CEN8*.[29] These genome edits ensures that each inner *CEN*-kinetochore complex is identical and can all be cleaved by designer Cas12 targeting guide RNAs (Figure 2). To increase the number of *CEN*-kinetochore complexes per cell, I will generate a series of polyploid *CentiCEN* strains using a new, rapid, and scalable mating type

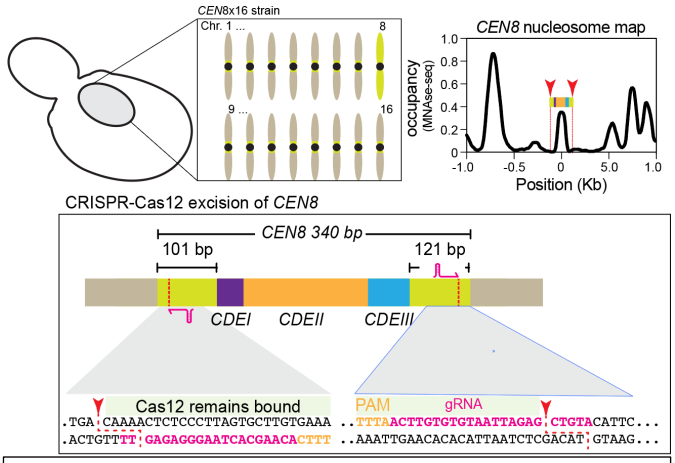



**Figure 2. Schematic Cas12-based kinetochore purification.** Each centromere is replaced by the *CEN8* fragment with targeting gRNAs within nucleosome free regions (data from Haase et al. 2023)

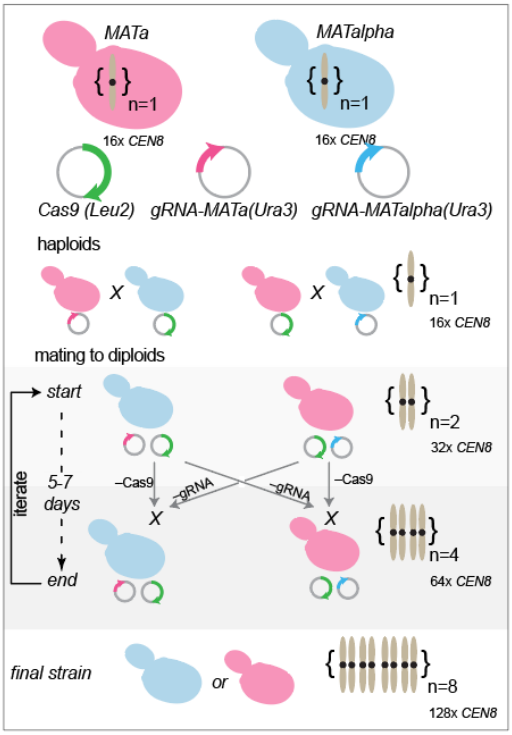



**Figure 3. Schematic of ICMBs for rapid generation of polyploid yeasts.**

switch method (Figure 3). By using Cas9-editing of the native MAT locus, Iterative-CRISPR-Cas9 By-MATing switch (ICBMs) will simultaneously switch the mating type and double the ploidy number. After removing either the guide or Cas9 plasmid, cells can then be immediately mated in successive rounds. Optimally, ICBMs will produce octaploid cells (8n) in as little as two weeks. ICBMs polyploidization will be monitored by cell size changes under optical microscope and by flow cytometry of DNA content. Any resulting polyploid clone will be robustly characterized for cellular fitness (growth rate, cell density, etc.). The total theoretical mass of kinetochores that can be isolated from an 8n *CentiCEN* strain is ~0.06 mg from 100 mL of a dense culture, compared to ~0.007 mg from the original haploid strain. Therefore, at least 6 liters of cells are required for cryo-EM, a manageable amount. WP2.B: Characterization and Cas12 solubilization of *CEN*-kinetochore complex. The rationale for choosing Cas12 to excise *CEN*-kinetochores is simple. While both Cas12 and Cas9 are single-turnover enzymes, Cas12 quickly releases the PAM-distal cleaved DNA while remaining bound to its target DNA, unlike Cas9, which firmly holds the two cleaved DNAs together.[30,31] Thus, two inverted Cas12-guide RNAs (gRNA) can be used to precisely excise *CEN*-kinetochore complexes from the bulk chromatin. In order to effectively isolate an endogenous *CEN*-kinetochore complex, I will ensure that Cas12 is effectively cutting the intended target. Guide RNAs will be selected to maximize on-target activity and minimize off-target activity using the CRISPick tool. Importantly, each gRNA will be positioned within a nucleosome-free region of the flanking *CEN8* DNA to ensure the target DNA is not blocked by nucleosomes (Figure 2). Cas12 cleavage activity will first be verified *in vitro* on a naked DNA template and then with isolated yeast chromatin. WP2.C: Solubilization and enrichment of endogenous *CEN*-kinetochore complexes. A gentle cell lysis approach will be used to limit disruption of natively chromatin-bound complexes. To this end I will use enzymatic cell lysis and permeabilization methods, such as Zymolyase combined with digitonin, to prepare the chromatin for Cas12 cleavage. As this process is done *in vitro* after harvesting cells, the action of DNA repair mechanisms is reduced. Thus, there will be little disruption the centromeric chromatin following Cas12 cleavage and we can further reduce any disruption via mild cross-linking with 0.5% paraformaldehyde. Additionally, there is no need for expression of Cas12 components in yeasts, reducing spurious cutting events prior to chromatin isolation. Solubilization of *CEN*-kinetochore complexes will be monitored by liberation of kinetochore proteins and *CEN8* DNA from the bulk chromatin using chromatin fractionation methods and assaying enrichment for proteins by western blot and *CEN8* DNA by Illumina sequencing. Finally, as Cas12 is FLAG-tagged, Cas12-bound *CEN*-kinetochore complexes can be enriched for by affinity purification.

### WP3. Structure of an endogenous yeast kinetochore

WP3.A: Single particle cryo-EM of an endogenous *CEN*-kinetochore complex. To obtain a sample suitable for structural work (e.g., homogenous and in sufficient quantity) we will use the *CentiCEN* strain. While natively 16 unique centromere sequences coordinate chromosome segregation, we make the assumption that *CEN8* is a sufficient representative of centromere-kinetochore complexes, generally. After rigorous testing of our Cas12-based purification method, purified *CEN*-kinetochore complexes will be inspected by negative stain EM to visualize if the purification methodology captures the overall inner kinetochore structure. Next, I will aim to obtain high-resolution structures using single particle cryo-EM on purified *CEN*-kinetochore complexes. Resulting models will be verified by construction of mutant binding interfaces and tested *in vivo* using plasmid shuffling. In particular, we will use the structure to investigate the topology of the *CEN* DNA. WP3.B: cryo-ET of endogenous kinetochore complexes. In tandem to the single particle studies, we will conduct structural studies *in situ* using the *CentiCEN* strain. *CentiCEN* yeast will be first prepared for cryo-ET by cryogenic focused ion beam (cryo-FIB) milling to produce thin lamella for imaging. To specifically target the kinetochore-spindle axis with cryo-fluorescence microscopy (cryo-FM), the *CentiCEN* yeast will be modified with an internal Cse4 GFP tag and an RFP tagged spindle pole body (Spc110). Data will be captured from multiple lamella and structures obtained with subtomogram averaging. Our goal is to achieve sub-nanometer resolution by using advanced tools available at MPI-Dortmund, such as the cryo-fluorescence light

microscope, the Thermo Scientific Hydra CX, and the Glovebox2.0, as well as using Alphafold2 and published structures for model fitting. Visualizing the kinetochore in its native configuration may reveal not only the centromeric interface but also how it interfaces with the spindle pole body. Mechanistic insights of these interfaces will be studied by using cell cycle arrest mutants in order to capture the native structure at key stages, including metaphase (p*MET3*-Cdc20), anaphase (cdc15-2), G1 (cdc6-1 or α factor), and hydroxy urea (early S-phase). This allows for an unparalleled temporal study of the kinetochore as it transits from Cse4$^{CENP-A}$ deposition to the end of chromosome division.

### *1.2.3 Gender dimension and other diversity aspects.*

*CENStruK* will be carried out at a Max Planck Institute (MPI), under the Max Planck Society (MPG). All MPG institutes adhere to the necessary requirements for supporting Gender Equality Plans, as confirmed by the letter of the Central Equal Opportunities Office. MPI–Dortmund is dedicated to creating a work environment free of gender bias, with Sigrid Rosin-Steiner, the Gender Equality Officer, actively advocating for MPI's Gender Equality Plans. I am fully committed to participating in workshops on gender issues and unconscious bias organized in our institute and offered by the MPG. This includes attending training courses on unconscious thought patterns, diversity, and discrimination, among other topics. MPI's Gender Equality Plans also fulfill the requirements set forth Horizon Europe. The planned research within *CENStruK* does not involve the study of gender or sex in biological systems. The model system of study is a single-celled fungus. While it possesses a complete sexual cycle with two mating types, which is used in *CENStruK*'s methodology, the extent to which its fungal mating system touch onto to themes of human gender, such as mating compatibility or gender, are beyond the scope of this research. Consequently, *CENStruK*'s inquiries into the structural aspects of the yeast kinetochore involves no human gender component.

### *1.2.4 Open science practices.*

*CENStruK* has been designed with a strong emphasis on open science practices. At the local level, the candidate will collaborate closely with two neighbouring Labs: Prof. Stefan Raunser's group for cryo-ET work, Prof. Boris Pfander's group for yeast work. My expertise in kinetochore biology and yeast genetics will complement the skills of trainees in both labs, especially advancing an open exchange of ideas and methods for cryo-ET with the Raunser group and cell cycle biology with the Pfander group. Furthermore, all laboratory notes generated during *CENStruK* will be stored and securely backed up using a third-party provider, Benchling, and made available upon request. One of the core goals of *CENStruK* is career advancement through the open sharing of its research output, thus all structural models will be openly shared prior to formal publication. This will be done by posting our findings to the pre-print repository bioRxiv alongside releasing access to the models on the Protein Repository Database. Once the method for Cas12-based purification is optimized, I will release a step-by-step protocol to the Protocols.io Platform. As *CENStruK* has benefited from the open sharing of reagents, I will advertise through my social media channels that the *CentiCEN* strain and any recombinant protein that are generated are available upon request.

### *1.2.5 Research data management and management of other research output.*

I will be fully responsible for the Findability, Accessibility, Interoperability, and Reusability of the proposed work. Integrity of raw data is critical to reproducible research. This necessitates its storage on at least two physically unlinked servers. Locally, data will be stored on the MPI-Dortmund "Billy" server with a backup in place on the MPG HPC server "Raven". Backups will be automatic on a weekly basis or when needed on demand (e.g. raw micrographs from cryo-EM). Additionally, metadata on the collection and analysis will be fully documented and secured. To achieve this, all documentation of data collection and analysis will be stored on the "Billy" server and the virtual Lab note book (Benchling). All yeast strains, bacterial plasmids stocks, and recombinant proteins will be documented and stored in a database with unique identifiers. Experimental data will be immediately analysed by myself and the interpretation will be presented during bi-annual group meetings, and at monthly subgroup meetings. Additionally, structural data analysis and management is to be done in conjunction with Dr. Marion Pesenti and Dr. Ingrid Vetter. All resulting raw data and structural models will be deposited to appropriate databases (SRA/GEO for sequencing datasets or PDB for atomic models). Any and all changes to our data interpretation will be documented within my laboratory notes and, for reproducibility, all biological and biochemical experiments will be done minimally in quadruplicate. Bi-monthly meetings will be done with Prof. Andrea Musacchio for the critical examination of experimental progress. Management of data used in final publications will be coordinated with the Max Planck Digital Library, which provides services to support scientists in research data management, such as the Edmond research data repository.

## *1.3 Quality of the supervision, training and of the two-way transfer of knowledge between the researcher and the host*

### *1.3.1 Qualifications and experience of the supervisor.*

Professor Andrea Musacchio will be the supervisor of *CENStruK*. Prof. Musacchio is an internationally recognized scientist for his fundamental work on elucidating the molecular mechanisms of the spindle assembly checkpoint and the molecular structure of the human kinetochore complex. His excellence is acknowledged by numerous awards including the endowed research Leibniz Prize (2020). He has led multiple international interdisciplinary collaborations and trained over 80 students and postdocs (e.g., Stefano Santaguida, Marina Mapelli, John Weir, Alex Faesen, Valentina Piano, Priyanka Singh, Pim Huis in 't Veld, Veronica Krenn, Luigi Nezi). As I have already enjoyed a *laissez faire* research environment during my PhD, my postdoc training will be structured similarly. In that regard I will make day-to-day experimental decision. However, bi-monthly one-on-one informal meetings will allow for open discussions of progress, ideas, and career development. Additional to ensure my success in acquiring the skills needed to do biochemistry, I will work closely with project group leaders (Dr. Marion Pesenti) and also in collaboration with Prof. Rausner Lab at the MPI-Dortmund.

### *1.3.2 Transfer of knowledge from the host to the researcher.*

A research training program is outlined below that will guide my professional development to launch my independent career. **Advance experimental and theory training**: Biochemistry: MPI-Dortmund and the Laboratory of Prof. Musacchio have decades of expertise on the cutting edge of biochemical techniques needed to solve biomolecular interactions. Being at MPI-Dortmund provides an unparalleled opportunity to learn several new techniques such as: pulldown assays, protein kinetics and binding, DNA topology assays, megadalton protein complex production, and cryo-EM/ET. Structural modelling: I will apply my already strong bioinformatic skills to learn the theory and practical aspects of structural modelling using cryo-EM datasets. This training will be assisted by Dr. Ingrid Vetter, the group's resident protein modeler. **Transferable skills development: Seminars and conferences:** I will attend upcoming conference on the molecular mechanism of chromosome segregation (e.g., GRC centromere) and evolution of cellular biology (e.g., Evo-chromo and Molecular mechanisms in evolution and ecology EMBO workshops) which will synergize my interests in evolution and developing skills of molecular mechanistic biology. I will apply to orally present my work (or as a poster) in order to continue building my international network. Lastly, I aim to publish my work in open access high visibility outlets as the fundamental insights into centromeric DNA function and technology development will be of high interest and impact. **Professional development:** MPI-Dortmund is an international center conducting high-quality interdisciplinary research, driven by a community of world-class collaborative scientists who share a vision of understanding the molecular mechanisms of biology. MPI-Dortmund is well positioned to prepare it trainees with career development courses and web series offered through the MPG "Training Center" web application (such as the "Building and Managing Your Research Group" workshop), which will prepare me to lead an international research group. Additionally, as a foreign resident in Germany, MPU-Dortmund offers German language courses (beginner to A2 level) which will greatly aid in my future navigation of a career in Germany. In collaboration with Prof. Musacchio, I will write perspectives pieces on articles which we peer-review and I will write a review article on the mechanism's centromere specification across life. These communication efforts will hone my compositional skills in order to effectively communicate my scientific work and ideas.

### *1.3.3 Transfer of knowledge from the researcher to the host.*

**Transfer of scientific expertise:** I bring nearly a decade of expertise in genetics, genomics and molecular biology to Prof. Musacchio's group, where the team's expertise lies at the intersection of biochemistry and cell biology. Specifically, though my genomics expertise, I will actively help in ongoing efforts to map $Cse4^{CENP\text{-}A}$ nucleosomes by sequencing methods. My experience with mega-base synthetic DNA construction projects (e.g., Sc2.0) will also provide valuable support as the group tackles the synthesis of challenging designer centromeric sequences. Furthermore, I will introduce the yeast model system to Prof. Musacchio's primarily mammalian-focused group. Expanding the group's focus to two evolutionarily distinct lineages will not only facilitate the transfer of methodologies (e.g., yeast genetics, strain construction) but also foster new evolutionary perspectives on the kinetochore and centromere. To support this collaboration between model systems, I will heavily participate in weekly group meetings by offering my suggestions, questions, ideas, and critiques. **Supervision skills:** Directly training students in Prof. Musacchio's group will accelerate knowledge transfer. Drawing from my past mentoring experience, I will advise PhD and Masters students during the course of *CENStruK*. Additionally, I will set aside

time for informal discussions with lab members to exercise their ideas and interpret their data. I will also offer guidance on setting up sequencing experiments and constructing phylogenetic trees. These activities will help me improve my supervision skills as I prepare to become an independent researcher in the near future.

### *1.4 Quality and appropriateness of the researcher's professional experience, competences and skills.*

Successfully implementing *CENStruK* requires a comprehensive understanding of centromere biology alongside sound technical skills. I have considerable experience in the fields of genetics, genomics, molecular biology, and evolution, which equips me with a unique skill set for investigating the molecular mechanisms of kinetochore structure and function in budding yeasts. During my PhD, I investigated the role of chromatin on kinetochore function, specifically detailing how mutant histones effected the function of the kinetochore. My PhD training therefore fostered a deep understanding of the yeast system and the biology of its centromere, which is an excellent base to begin my structural mechanistic work. My broad interests and past training in scientific topics, from the ecology and evolution of metabolic gene clusters to mechanisms of chromosome segregation, opens up the possibility for making connections between traditionally separate fields. Furthermore, I have a proven track record for implementing research projects and delivering results, as evidenced by my 18 publications in respected international journals, including four first-author papers from my PhD alone. This demonstrates my effectiveness as a communicator and productive member in my field.

## 2. Impact #@IMP-ACT-IA@#

### *2.1 Credibility of the measures to enhance the career perspectives and employability of the researcher and contribution to his/her skills development.*

I aspire to apply for assistant professorships upon completing my postdoctoral training, with the goal of managing my own research group in mechanistic evolutionary biology. The skills I will acquire in Prof. Musacchio's group, particularly in cryo-EM and cryo-ET techniques, will make me a competitive applicant for these positions. Specifically, the experience of setting up innovative methods for biochemical and structural studies will compliment my strong background in genetics and evolutionary biology. I will receive thorough training in cryo-ET, including sample preparation and data analysis, in conjunction with the Rausner Lab at MPI-Dortmund, who are world leaders in this technique. Additionally, since the host lab does not currently work with yeast, I am launching a new research program within the lab. This experience will be a model that I can use in the future when starting my independent research group. Therefore, the MSCA fellowship will be an unequalled experience that builds my scientific qualifications to enhance my future employability. As a recent immigrant, I currently do not have a robust scientific network within the EU. As I plan to remain in the EU due to my established family ties, being part of the MSCA fellowship and its alumni network offers an exciting opportunity to expand my scientific connections in my new home. Thus, the fellowship is a crucial stepping stone toward establishing my independence in the EU.

### *2.2 Suitability and quality of the measures to maximise expected outcomes and impacts, as set out in the dissemination and exploitation plan, including communication activities.* #@COM-DIS-VIS-CDV@#

Advances in science relies on making scientific output accessible to a range of audiences, from peers to the public. Therefore, I envision several opportunities to maximize dissemination of *CENStruK*'s scientific output. To provide early access and visibility of our research findings, we will preprint all publications to bioRxiv prior to formal publication. Additionally, each bioRxiv document will be updated based on valuable feedback from colleagues and peer review, improving the quality of *CENStruK*'s research. We will aim to publish in prestigious international journals to maximize both the visibility and impact of *CENStruK*'s research. Importantly, we will provide an open peer-review file for each publication in order to help young trainees and the public understand the research process and the quality control involved. *CENStruK*'s publications will be broadcasted by various channels, including MPI-Dortmund's official social media accounts (Twitter/X), MPG (Max-Plank Gesellschaft) press releases, and through the personal social media accounts of Prof. Musacchio and Dr. Haase, to maximize the reach to diverse audiences and increase research visibility. In addition to increasing the visibility of the final research output, we will present *CENStruK*'s in-progress and unpublished data at conferences, both orally and as posters, to gather feedback and foster serendipitous collaboration. To increase visibility with the local community and to showcase MPI-Dortmund's research, I will actively participate and help organize the yearly "Open Day/Tag der offenen Tür", in which Dortmund locals have firsthand access to the Institute and its cutting-edge science. Moreover, MPI-Dortmund's public relations office will play a key role in making *CENStruK*'s research findings accessible and understandable to a general audience. Intellectual property management will be handled by the MPG office for technology transfer (Max Planck Innovation), which facilitates the commercialization of research outcomes to benefit society.

### *2.3. The magnitude and importance of the project's contribution to the expected scientific, societal and economic impacts.*

**Expected scientific impact:** The nature of the centromeric nucleosome in the budding yeast has long been sought and debated over. Thus, the immediate impact of solving an endogenous structure for the inner kinetochore will be to settle this long-standing debate definitively. This will provide new knowledge for scientists who study centromeres and kinetochores, chiefly explaining how centromeric DNA assembles with the inner kinetochore. Moreover, a detailed atomic model of the endogenous structure will provide a complete rationale for why the CENP-A histone is essential for kinetochore assembly, which will not only be influential within the yeast community, but also for kinetochore biology as a whole. From the perspective of basic biology, the potential magnitude and importance of *CENStruK's* output will be of general interest to the broader field of cell cycle biology. A deep mechanistic understanding how centromeric DNA assembles the inner kinetochore will also be of great interest to those in translation research, as errors in segregating the genetic material can cause developmental damage and abnormalities (e.g., cancers and Down syndrome). **Expected economic, technological, and societal impact:** Technology stemming from this work, such as the Cas12 affinity purification of chromatin-bound complexes, will be a broad interest beyond the fields of kinetochore and chromosome biology. For example, the Cas12-based affinity purification will enable native purification of RNAP complexes or histone-modifying complexes. Thus, *CENStruK* is expected to contribute both fundamental biological insights and tangible technologies, which will promote EU scientific excellence, innovation, and competitiveness.

#§COM-DIS-VIS-CDV§# #§IMP-ACT-IA§#

## 3. Quality and Efficiency of the Implementation #@QUA-LIT-QL@# #@WRK-PLA-WP@# #@CON-SOR-CS@# #@PRJ-MGT-PM@#

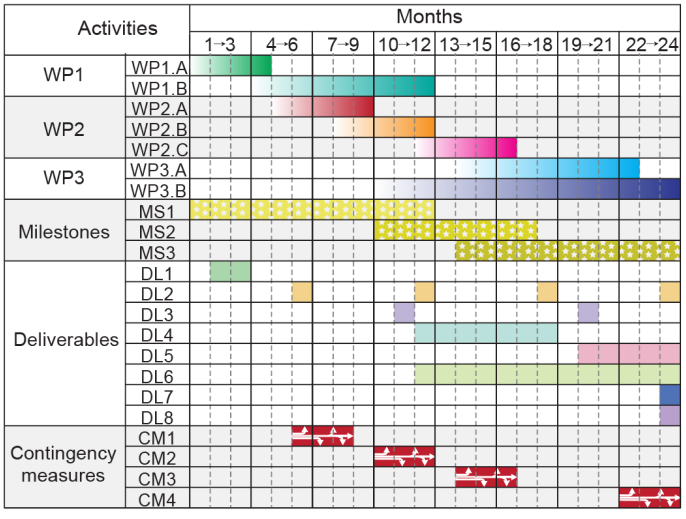
*Acronyms for this section:* **MH**: Max Haase, **AM**: Andrea Musacchio, **MP**: Marion Pesenti, **MS**: MPI Dortmund Staff Scientist(s), **SR**: Prof. Stefan Raunser.

### *3.1 Quality and effectiveness of the work plan, assessment of risks and appropriateness of the effort assigned to work packages*

#### *3.3.1 Milestones.*

MS1. Purification and reconstitution of the Hsp90-dependent assembly of the yeast inner kinetochore. MS2. Construction of the *CentiCEN* strain and Cas12-based kinetochore-CEN purification. MS3. *In situ* structural studies of the *CentiCEN* strain using cell cycle arrest mutants.

#### *3.1.2 Work Packages.*




**WP1. Reconstitution of the yeast inner kinetochore.** WP1.A (months 1-4): Expression and purification of the yeast inner kinetochore. **MH** with the help of **MS** will overexpress needed proteins using bacterial and insect cell systems and purified proteins quality will be assessed using the **PCF** at **MPI**. WP1.B (months 4-12): Reconstitution of the Hsp90-liscening of the active Cbf3-*CEN* complex. **MH** will conduct binding assays to assemble the yeast inner kinetochore with the Hsp90 activation step for cryo-EM [**MH** in supervision with **MP**]. **WP2. Construction of the *CentiCEN* yeast strain and Cas12-based kinetochore purification.** WP2.A (months 5-9): Polyploid *CentiCEN* strain construction. **MH** will validate the ICBMs methodology and generate a series of polyploid strains. **MH** will conduct all validation steps (FACs, phenotypic growth assays, etc.). WP2.B (months 8-12): Characterization and Cas12 solubilization of kinetochore-*CEN* complex. **MH** will carry out Cas12 cleavage validation experiments on naked DNA and native yeast chromatin. **MS** will purify Cas12 for *in vitro* cleavage. WP2.C (months 12-16): Solubilization and enrichment of endogenous kinetochore-*CEN* complexes. **MH** will purify endogenous kinetochore-*CEN* complexes from isolated yeast nuclei [quality control with **PCF**]. **WP3. Structure of an endogenous yeast**

**kinetochore** WP3.A (months 14-22). Single particle cryo-EM of an endogenous kinetochore-CEN complex. **MH** in supervision of **MP** will examine purified endogenous kinetochore-*CEN* complexes by cryo-EM. WP3.B (months 10-24). cryo-ET of endogenous kinetochore complexes. **MH** with the help of **MS** and in supervision of **AM** and **SR** will carry out *in situ* characterization of kinetochores from the *CentiCEN* strain.

### *3.1.3 Deliverables.*

Deliverables will be prepared by **MH** with the guidance of **AM**. DL1 (months 2&3): Design of project/career development plan; DL2 (at months 6, 12, 18, 24): Progress report on key results and critical evaluation project progress. DL3 (months 11&20): Present work at international conferences. DL4 (months 12-18): Write review article on centromere and kinetochore evolution in fungi. DL5 (months 20–24): Write up data for manuscript submission(s) to high-impact international journals. DL6 (months 12-24): I will conduct public engagement of our science via twitter updates. DL7 (months 24): Dissemination of publication(s) through Max Planck Society press release. DL8 (month 24): Career plan update and prepare portfolio for applying for PI positions.

### 3.1.3 *Appropriateness of the effort assigned to work packages.*

The time dedicated to *CENStruK* is 24 months. **MH** is helped by three full time research scientists, whom collectively have >60 years' worth of experience in biochemistry and are actively integrated to achieve the **WPs** aims. **WP1**: MPI provides all instrumentation and consumables needed for recombinant protein expression, biochemical binding assays, and cryo-EM sample preparation and data collection. Both the local compute and the MPG HPC server "Raven" will be used for cryo-EM data analysis. Additionally, all purified proteins are expertly quality checked by the **PCF. WP2**. **MH** has a decade of experience in strain construction and all required reagents are available, or will be made available, at the **MPI**. The *CEN*-kinetochore purifications will be carried out by **MH** with assistance from **MP**. **WP3**. All instrumentation and reagents needed to carryout cryo-EM and cryo-ET are at **MPI**. As a part of **MH**'s skill development, he will be instructed in the use of these instruments by **MP** and **SR**. For cryo-ET work, **SR** has advanced protocols for the preparation and processing of yeast cells including technicians to accelerated the progress of data acquisition.

### *3.1.5 Risk Assessment.*

**Contingency measures** (**CMs**) are to help mitigate risks associated with *CENStruK* and are addressed under the different **CMs**. **Risk 1:** Hsp90-Sgt1 is not the sole factor for Cbf3c activation. CM1: It is unlikely that Hsp90-Sgt1 will not function to activate Cbf3c, although we cannot rule out the possibility that other partners are involved in this process that we are not recombinantly expressing. In this case we will generate whole cell yeast lysates that contain the Hsp90 function and use sub-stoichiometric amounts to activate recombinant Cbf3c. **Risk 2:** Polyploids may be unstable CM2: A consequence of polyploidization is the opportunity for whole chromosomes to be lost since they are no longer individually essential, consequently this could bias expression of various kinetochore components. In this case we carefully characterize our strains by whole genome sequencing and RNA-seq in order to understand the extent of chromosome instability and select clones with the least amounts of changes. Lastly, we would move forward with a stable diploid or tetraploid strain or an ectopic plasmid-based system for centromere amplification. **Risk 3:** *CEN8*-Kinetochore may not solubilize CM3: Solubilization by Cas12 cleavage is expected to be robust as it is conceptually similar to the CUT&Run methodology and yeast kinetochores can be routinely isolated by more violent lysis methods. However, kinetochores could remain bound to spindle microtubules given our gentle lysis, in this case we will either add microtubule depolymerizers (e.g., nocodazole) or implement a TEV cleavable Ndc80 to severe microtubule connections. **Risk 4:** cryo-EM and cryo-ET pitfalls CM4: During sample preparation we may face the issue that *CEN8*-kinetochores are not be stable on grids. In this event we will apply mild cross-linking to the sample (e.g., GraFIX), which has been successful for the human CCAN complex. Additionally, we could also use small LEA proteins, which provide a simple and cost-effective method to overcome air-water-interface damage to sensitive cryo-EM samples.[32] Lastly, we may have contaminating ice on our milled lamella for cryo-ET. In this event I will work closely with experts in the Rausner Lab to mitigate ice formation (e.g., use of the "Glove box 2.0").

## *3.2 Quality and capacity of the host institutions and participating organisations, including hosting arrangements.*

### *3.2.1 Hosting arrangements.*

MPI Dortmund has a storied history and is a lauded institute of the Max Plank Society. Together, a diverse group over 200 scientists from over 30 countries with backgrounds in disciplines spanning chemistry, biology,

biochemistry, and theoretical chemistry/biology work to understand the molecular mechanisms of life. The institute is grouped into three departments – Mechanistic Cell Biology, Structural Biochemistry, and Systemic Cell Biology – which collaborate to address both fundamental questions of cell biology and to develop potential therapeutic strategies towards disease treatment. The Mechanistic Cell Biology (MCB) department of Prof. Andrea Musacchio host ~50 researchers at every level of training, including the project leaders Drs. Maffini, Pesenti, and Vetter. The rally cry of the MCB is to wholistically understand the molecular mechanisms that ensure correct inheritance of the genetic material during cellular division. Having spent over two decades leading a research group and a MPI department, **AM** is a proven world class mentor with high rates of success of his postdoctoral trainees. He is encouraging of inter-departmental collaboration and has set a scientific culture that is friendly, rigorous, and innovative. **AM** will provide financial support to cover research costs and training in biochemistry and structural biology.

### *3.2.2 Quality and capacity of the host institutions and participating organizations.*

MPI-Dortmund has world-class facilities for cutting edge structural mechanism research. This is possible through extensive core funding from the Max Planck Society and external funders such as the ERC, DFG, and the Boehringer Ingelheim foundation. MPI-Dortmund has a reputation of providing a strong foundation for trainees to achieve academic and professional excellence. The Institute has continually recruited competitive researchers from across the globe to Dortmund and guarantees treatment according to the Charter of Fundamental Rights of the EU during their entire stay in MPI. Additionally, MPI Dortmund upholds the equal rights and opportunities standards according to MPG equality of opportunities regulation irrespective of sexual identity, gender, colour, and religion. MPI Dortmund has a Grant office that assists scientists in writing proposals. With extensive experience in European projects, such as currently held ERC grants, the Institute provides full administrative and financial support to scientists. Additionally, it ensures that all scientific activities comply with the European Charter for Research. The Department of MCB spans two floors of fully furnished laboratories for biochemistry, molecular biology, cell biology and structural biology. This includes facilities such as multiple ÄKTA Pure systems, a collection of >200 antibodies, a collection of recombinant proteins involved in cell cycle processes (e.g., Aurora B, Hsp90-Sgt1, fully active PLK1, CDK1/Cyclin A, etc.), suites for cell culture (bacteria, yeast, mammalian), and multiple microscopes, such as DeltaVision Elite and Zeiss Axio Observer Z1 Advanced. Additionally, through the Electron Microscopy Core, access to the necessary facilities for cutting-edge cryo-electron microscopy is provided (e.g., FEI Titan Krios EM and a 300kV cryo-EM JEOL JEM 3200FSC). The MCB also provides personal workstation and office for each researcher to facilitate their research, reading, and writing. MPI provides access to over 2000 journals through the Max Planck Society. MPI also licences many scientific software packages including Prism, PyMol, Adobe, Origin, and Endnote. In addition, the IT Service Facility assists with software acquisitions, installation, and troubleshooting.

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