Nuclear pore complexes (NPCs) are the main conduits for molecular exchange across the nuclear envelope. The NPC is a modular assembly of ~500 individual proteins, called nucleoporins or nups. Most scaffolding nups are organized in two multimeric subcomplexes, the Nup84 or Y complex and the Nic96 or inner ring complex. Working in *S. cerevisiae*, and to study the assembly of these two essential subcomplexes, we here develop a set of twelve nanobodies that recognize seven constituent nucleoporins of the Y and Nic96 complexes. These nanobodies all bind specifically and with high affinity. We present structures of several nup-nanobody complexes, revealing their binding sites. Additionally, constitutive expression of the nanobody suite in *S. cerevisiae* detect accessible and obstructed surfaces of the Y complex and Nic96 within the NPC. Overall, this suite of nanobodies provides a unique and versatile toolkit for the study of the NPC.
The hallmark of the eukaryotic cell is a complex endomembrane system of organelles that compartmentalize specific functions within the cell. One of the largest of these organelles is the nucleus, which stores the genetic material and is the site of replication, transcription, and ribosome synthesis. Soluble transport of molecules into and out of the nucleus occurs solely through nuclear pore complexes (NPCs), ~40–120 MDa ring-like channels that perforate the inner and outer membranes of the nuclear envelope (NE) (Fig. 1a). Roughly 30 nucleoporeins (nups) contribute to the modular, eightfold symmetric assembly of subcomplexes that comprise the NPC1-4. One of the main structural subcomplexes is the Nup84 Y complex. In S. cerevisiae, the 575 kDa Y complex has seven components, many of which are essential or produce severe phenotypes when deleted5–7. The Y complex structure consists of two short arms that meet a long stalk at a central triskelion-like hub8–10 (Fig. 1b).

The other main structural component of the NPC is the hetero-meric Nic96 or inner ring complex11. This ~0.5 MDa complex occupies the inner ring of the NPC and anchors the trimeric Nsp1 complex, which contains three phenylalanine-glycine (FG)-nups that are essential for maintaining the permeability barrier11–16. Both Y complex nups (Nup84, Nup85, and Nup145C) and Nic96 have an ancestral coatamer element 1 (ACE1) fold, conserved across the NPC and COPII vesicle coats17,18. The tripartite fold consists of crown, trunk, and tail modules and the interfaces between each module act as somewhat flexible joints. How this flexibility affects the NPC assembly is still unclear.

Its size, flexibility, and membrane interactions pose challenges for the elucidation of the structure of the NPC. But only with detailed structural information will we obtain mechanistic insight into the many functions of NPCs. To arrive at a structure, different labs approach the problem by either a bottom-up or top-down approach. For the bottom-up approach, the many modular structural assemblies that make up the NPC have been broken down into further sub-assemblies which are then studied primarily by X-ray crystallography. Over the past decade, many of the structural elements have been characterized (reviewed in refs. 2,19). We now have a complete composite model for the Y complex from S. cerevisiae20. For the top-down approach, cryo-electron tomography (cryo-ET) has been used to visualize whole NPCs while still embedded in the nuclear membrane or after detergent extraction. Recent studies have yielded maps of the entire NPC at 2–5 nm resolution for human, Xenopus laevis, and S. cerevisiae21–24. The resolution gap between the top-down and bottom-up approaches has narrowed. Along with multiple stochiometry studies, docking of the many crystal structures of nups into the cryo-ET maps has been attempted22,23,25–28. For S. cerevisiae, the cryo-ET map allows placement of Y complexes into the density, resulting in a model that contains a total of 16 copies per NPC24. This model consists of two eight-membered rings, one each on both the cytoplasmic and nucleoplasmic faces of the NPC23,24. The Y complexes are arranged in a head-to-tail manner, with the main interface mediated by Nup120 and Nup13329. On the cytoplasmic face, the Nup82 complex anchors to the Y complex via Nup8530. Thirty-two copies of Nic96 are nestled tightly into the inner ring complex along with Nup192, another scaffolding nup33. While these studies improved our understanding of the overall architecture of the NPC, the resolution of these cryo-ET maps does not reveal secondary structure. This leaves room for further interrogation and improvement. We aimed to create a set of tools that will aid in studying the complicated NPC assembly in more detail.

Here we describe a nanobody library comprising 12 unique nanobodies to the Y complex and Nic96 from S. cerevisiae. Nanobodies are single-domain (VHH) antibody fragments derived from camelid heavy-chain only antibodies. Nanobodies are excellent tools both in vitro and in vivo, as they are small (~14 kDa), easily purified from E. coli, easily modified with fluorophores, and typically have nanomolar binding affinities31,32. The library consists of nanobodies that bind to each of the 6 conserved nups in the Y complex3. We describe their in vitro binding characteristics using bio-layer interferometry, size-exclusion chromatography (SEC), and, in several cases, their nup-bound structures by X-ray crystallography. We show the effects of nanobody expression in vivo and how these results suggest accessible and inaccessible surfaces within the assembled NPC. Together, this work provides a toolkit for studying the scaffold of the NPC and uncovers details of the NPC structure in vivo.

Results

A nanobody library to the Y complex and Nic96. In order to generate nanobodies specific to NPC scaffold nups, we separately immunized alpacas with recombinantly purified full-length Y complex and Nic96. We then selected nanobodies by phage display using single nups or subassemblies of the Y complex as targets (Fig. 1b). This allowed us to obtain nanobodies that cover both short arms, the hub, and the long stalk of the Y complex. We thus compiled a set of twelve nanobodies that bind Y complex nups and Nic96 (Fig. 1b, c). The library covers a wide range of sequence space (Fig. 1c). Both, the sequences and lengths of complementarity determining regions (CDRs) 1 and 2, are more similar than CDR3 across the library. However, there is no common CDR across the set. The greatest differences arise in CDR3, which varies from 9 to 24 residues. This wide deviation in length is attributed to the vast genetic diversity contributed by the immunized alpaca, rather than the typically shorter and invariant lengths used in in vitro selection methods33,34.

After selection, we obtained two nanobodies (VHH-SAN6 and 7) that recognize a truncated Y complex hub construct (Nup120 and Nup85 C-terminal domains, full-length Nup145C-Sec13) (Fig. 1b). In order to identify the nups these nanobodies target, we first tested binding by SEC with full-length nups (Nup120, Nup85, and a fusion construct of Nup145C-Sec1335). We found that both bound Nup145C-Sec13. To further narrow down the binding site, we conducted a second SEC experiment using only Sec13 fused to the Nup145C insertion blade35 and found that VHH-SAN7 recognizes Sec13 rather than Nup145C (Supplementary Fig. 1). VHH-SAN6 did not bind Sec13 alone, meaning that it recognizes Nup145C. Sec13 is tightly packed within the hub of the Y complex, so we tested the ability of VHH-SAN7 to bind the assembled Y complex hub. When pre-incubated with Nup120-Nup85-Seh1-Nup145C-Sec13, VHH-SAN7 co-eluets as a heterohexameric complex (Supplementary Fig. 1). This experiment suggests that VHH-SAN7 binds Sec13 in the context of the assembled Y complex.

Three nanobodies (VHH-SAN1/2/3) in the library recognize Nup85. As an ACE1 nup, we examined whether these nanobodies bind to one of three modules (crown, trunk, tail) within the domain. By SEC analysis we found that all three nanobodies bound Nup85-crown نNup85-crown-Seh1 (Supplementary Figs. 2, 3). We then tested the binding of each nanobody to Nup85-crown, which formed a stable complex with VHH-SAN2 and 3, but not VHH-SAN1 (Supplementary Fig. 2). Binding data suggest that VHH-SAN2 and 3 recognize distinct, non-overlapping epitopes, as we observe a heptameric complex of Nup85-crown-Nup85-crown-Nup85-crown-Nup85-crown-Nup85-crown-Seh1-VHH-SAN2/3 by SEC (Supplementary Fig. 2). We verified that VHH-SAN1 bound Nup85 by observing no complex formation with Seh1 alone (Supplementary Fig. 3).

Nanobodies bind with varying kinetics, but strong affinities. In order to characterize the binding kinetics of our nanobody

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library, we employed bio-layer interferometry (BLI). We affixed each nanobody with a C-terminal biotinylated Avi-tag\textsuperscript{36} to streptavidin-coated biosensor tips. We assayed binding to each nanobody’s respective nup target and observed a variety of binding kinetics across the nanobody library (Table 1, Fig. 2). The tightest binder, VHH-SAN3, dissociates very slowly resulting in a binding constant of \(~14\) pM. In fact, a slow dissociation rate applies to the majority of the library. A few nanobodies,
VHH-SAN2, 3, 8, and 9, have off rates less than \(1 \times 10^{-4} \text{s}^{-1}\) resulting in very tight equilibrium binding constants (14–170 pM). Of the set, VHH-SAN4 has the comparatively weakest affinity of \(\approx 230 \text{nM}\), due to a much faster off rate than the other members of the library and is the only nanobody that has an equilibrium binding constant of less than \(10 \text{nM}\). Overall, the nanobodies bind very stably to their antigens.

Mapping of nanobody epitopes by X-ray crystallography. We mapped the epitopes of eight of the twelve nanobodies by solving the crystal structures of multiple nup-nanobody complexes. This enabled us to visualize the binding epitopes in greater detail. We used as analytes. Curves were corrected for buffer background. Each set of curves is a twofold dilution series from the highest concentration listed on each plot. Experimental data are shown as black dotted lines with red lines indicating globally fitted curves. Axis labels for all plots are indicated in the bottom left.

### Table 1 Nanobody-binding affinities.

| Nanobody | Nucleoporin | \(k_D\) (M) | \(k_{on}\) (M \(^{-1}\text{s}^{-1}\)) | \(k_{off}\) (s \(^{-1}\)) |
|----------|-------------|-------------|-----------------|-----------------|
| VHH-SAN1 | Nup85       | \(5.8 \times 10^{-10}\) | \(2.3 \times 10^{5}\) | \(1.4 \times 10^{-3}\) |
| VHH-SAN2 |             | \(8.0 \times 10^{-11}\) | \(1.0 \times 10^{6}\) | \(8.1 \times 10^{-5}\) |
| VHH-SAN3 |             | \(1.4 \times 10^{-11}\) | \(5.8 \times 10^{5}\) | \(8.1 \times 10^{-6}\) |
| VHH-SAN4 | Nup133      | \(2.3 \times 10^{-7}\) | \(9.5 \times 10^{5}\) | \(8.2 \times 10^{-1}\) |
| VHH-SAN5 |             | \(1.0 \times 10^{-8}\) | \(2.8 \times 10^{5}\) | \(8.2 \times 10^{-3}\) |
| VHH-SAN6 | Nup145C     | \(2.4 \times 10^{-10}\) | \(5.3 \times 10^{5}\) | \(1.3 \times 10^{-4}\) |
| VHH-SAN7 | Sec13       | \(3.1 \times 10^{-10}\) | \(3.5 \times 10^{5}\) | \(6.5 \times 10^{-4}\) |
| VHH-SAN8 | Nup84       | \(6.3 \times 10^{-11}\) | \(3.5 \times 10^{5}\) | \(2.2 \times 10^{-5}\) |
| VHH-SAN9 |             | \(1.7 \times 10^{-10}\) | \(4.4 \times 10^{5}\) | \(7.7 \times 10^{-5}\) |
| VHH-SAN10 | Nup120     | \(3.8 \times 10^{-10}\) | \(4.5 \times 10^{5}\) | \(1.7 \times 10^{-4}\) |
| VHH-SAN11 |             | \(7.9 \times 10^{-10}\) | \(9.8 \times 10^{5}\) | \(7.8 \times 10^{-4}\) |
| VHH-SAN12 | Nic96       | \(1.3 \times 10^{-9}\) | \(1.8 \times 10^{6}\) | \(2.4 \times 10^{-3}\) |

**Fig. 2 Bio-layer interferometry of nanobody-nup binding.** a Schematic of the Y complex and Nic96 with the nanobody library. b Binding curves showing association and dissociation kinetics for each nanobody-nup pair. Nanobodies with a biotinylated C-terminal Avi-tag were used as analytes. Curves were corrected for buffer background. Each set of curves is a twofold dilution series from the highest concentration listed on each plot. Experimental data are shown as black dotted lines with red lines indicating globally fitted curves. Axis labels for all plots are indicated in the bottom left.
of positional refinement, the nanobody fit reasonably well into the density (Supplementary Fig. 4).

**VHH-SAN10 recognizes a flexible loop in Nup120.** We also solved the complex of Nup120\textsubscript{186–757}–VHH-SAN10/11 by MR using the published scNup120\textsubscript{186–757} structure as a model.\cite{46}. After initial refinement, we observed additional density near residues 431–439 of Nup120, which we could attribute to a nanobody using MR. However, there was no obvious additional density for a second nanobody. Analysis of the crystals by SDS-PAGE showed that both nanobodies were present in the crystal (Supplementary Fig. 5). We then examined the crystal packing to see if there was space to accommodate the missing nanobody adjacent to unstructured loops. We hypothesized that if the nanobody was completely missing in the density, the epitope was also disordered in the crystal and therefore missing in the density as well. There are five unstructured loops in the β-propeller domain of Nup120 that neighbor solvent channels (Supplementary Fig. 5). A nanobody’s dimension are roughly 35 Å × 15 Å × 15 Å. Only one large loop, residues 187–203, faces a solvent channel in the crystal that appeared sufficiently large to accommodate a nanobody. We hypothesized that the missing nanobody bound this loop and tested whether replacement of residues 187–203 with a flexible linker (GGSx5) would ablate binding by BLI. Indeed, we found that VHH-SAN11 no longer bound this Nup120 mutant, but as expected, VHH-SAN11 still recognized Nup120 (Fig. 4c). In a reciprocal experiment, we replaced residues 431–439 with a flexible linker (GSSx3) and tested VHH-SAN10 versus VHH-SAN11 binding. This experiment confirmed that VHH-SAN11, and not VHH-SAN10, binds to the 431–439 region (Fig. 4b).

Both VHH-SAN10 and 11 are interesting nanobodies, as they recognize relatively unstructured regions of Nup120. Typically, nanobodies recognize structured regions or clefts in their target, owing to their long CDR loops that prefer to insert into and along concave surfaces. Regardless of this difference, both have very high binding affinities (Table 1). VHH-SAN10 and 11 add to a growing list of nanobodies that bind to short epitopes that function outside of a folded domain.\cite{41,42,43,44}.

**VHH-SAN12 binds Nic96 between its trunk and tail modules.** In addition to the Y complex nanobodies, we identified a nanobody, VHH-SAN12, that binds Nic96 of the inner ring complex. We solved the complex of Nic96\textsubscript{186–839} with VHH-SAN12 by MR with the published scNic96\textsubscript{186–839} structure as a template (Fig. 5a).\cite{45}. Nic96\textsubscript{186–839} forms an elongated structure of 30 α-helices. Overall, the dimensions, shape, and ACE1 fold of Nic96 are identical to the previously described structures.\cite{45,46}. The N terminus is in the center of the protein that then zig-zags towards one end of the molecule. Helices α4–12 fold over themselves, forming the crown of the ACE1 domain, with α6–9 running perpendicular to the trunk helices of α13–21. The C-terminal helices α22–30 form the tail and zig-zag away from the trunk at an angle. VHH-SAN12 inserts its CDR loops 1 and 2 into the space between helices α20–21 and α22–25. Interestingly, VHH-SAN12 has one the shortest CDR3 of the library and CDR3 contributes little to the binding interface, which is unusual for a nanobody.\cite{37}. Even without this contribution, VHH-SAN12 has a high affinity for Nic96 (Fig. 2, Table 1).

Helices α20–25 delineate the trunk and tail interface of the ACE1 fold. In comparison to the previously solved structures, we observe a change in conformation. Aligning the tail domains of this structure and the structure from Jeudy et al.\cite{45}, we observe a kink that translates through the remainder of the molecule (Fig. 5b). VHH-SAN12 brings helices α20–21 and α22–25 slightly closer to each other, resulting in a ~19 Å shift between the two crown domains. This shift is slightly smaller (~10 Å) when comparing our structure to the one described in Schrader et al.\cite{46}. While the overall conformation changes, the three modules of the ACE1 fold individually superpose very well. In comparing the crown domains, our new structure is much better defined (Fig. 5c). At the increased resolution (from 2.5 to 2.1 Å), we were able to complete Nic96 by including helix α9, and two loops including 30 additional residues. We speculate that the improvement of the data is due to the ability of the nanobody to stabilize Nic96 in one conformation. Either this is an effect of the nanobody alone, or it may be a combination of nanobody-binding paired with crystal packing.

**Several NPC nanobodies localize to the NE in vivo.** Having characterized this nanobody library in vitro, we asked whether these nanobodies would bind their targets in vivo and whether their expression would affect cellular fitness. We first put the
production of each nanobody under control of the high expression GAL promoter in a wild type yeast strain. We observed no fitness defects upon nanobody expression at both 30 °C and 37 °C (Fig. 6a). Given the small size of a nanobody relative to the NPC, this was not entirely surprising, but also suggests that none of the surfaces occupied by the nanobodies are essential for NPC integrity, assuming that the nanobodies can find their targets in the cell. We therefore asked whether the nanobodies localized to the NPC and the NE in vivo, thus examining their ability to bind their targets within the context of the assembled NPC. To this end, we fused mKate2, a monomeric far-red fluorescent protein 40, to the C terminus of each nanobody. We then expressed these nanobody-mKate2 fusions in a yeast strain with endogenously expressing Sec13 in the COPII vesicle coat or SEA complex, rather than forming a complex with the copies of Sec13 in the NPC. 47–49. We suggest that binding of VHH-SAN7 to these cytoplasmic copies of Sec13 may also account for the fitness defect of this strain. The majority of cells expressing both Nup84 nanobodies (VHH-SAN8 and 9), also showed strong nuclear rim localization with Nup120. However, VHH-SAN9 had a curious effect on Nup120 localization. While some cells expressing VHH-SAN9 showed strong colocalization with Nup120-GFP, many other cells showed reduced rim colocalization. VHH-SAN4 and 5 nanobodies, which recognize Nup133 at its N-terminal β-propeller, did not colocalize with Nup120 but instead were distributed diffusely throughout the cell. VHH-SAN6, which recognizes Nup145C in the Y complex hub, also strongly enriched with Nup120 at the nuclear rim. VHH-SAN7 formed puncta in the cytoplasm, possibly due to binding Sec13 in the COPII vesicle coat or SEA complex, rather than forming a complex with the copies of Sec13 in the NPC 47–49. In the absence of any nanobody expression, we observed clear nuclear rim fluorescence for Nup120-GFP, while mKate2 alone produced diffuse fluorescence throughout the cytoplasm and the nucleus (Fig. 6b, ‘Mock’). Of the three Nup85 nanobodies, one exhibited strong co-localization with Nup120 at the nuclear rim (VHH-SAN3) while the other two (VHH-SAN1 and 2) displayed reduced rim colocalization. VHH-SAN4 and 5 nanobodies, which recognize Nup133 at its N-terminal β-propeller, did not colocalize with Nup120 but instead were distributed diffusely throughout the cell. VHH-SAN6, which recognizes Nup145C in the Y complex hub, also strongly enriched with Nup120 at the nuclear rim. VHH-SAN7 formed puncta in the cytoplasm, possibly due to binding Sec13 in the COPII vesicle coat or SEA complex, rather than forming a complex with the copies of Sec13 in the NPC 47–49.
equilibrium binding constant of <10 nM. Through both SEC and X-ray crystallography, we mapped their general binding sites or epitopes. The recognized epitopes range from relatively unstructured loops to clefts between domain interfaces. We also expressed these nanobodies in yeast cells to assess their ability to localize to NPCs in their native cellular environment. Armed with this information, we can now interpret the heterogeneity of the observed in vivo effects and identify elements of this library that can aid in future NPC assembly studies.

The Nup85 crown is thought to be adjacent to the Nup82 complex, where both VHH-SAN2 and 3 bind24,30 (Fig. 7a, b). This correlates well with the localization of VHH-SAN2 being largely diffuse throughout the cell, suggesting that the VHH-SAN2 binding site is occluded. However, there is still some enrichment at the NE with Nup120, suggesting the Nup82-Nup85 tether may be flexible or dynamic, allowing for a fraction of the nanobody pool to still bind. Another possibility is that some Nup85 epitopes may be accessible, while others are not. This would suggest that Nup85 is differently assembled in different parts of the NPC. Both VHH-SAN1 and VHH-SAN3 are strongly enriched at the NE. However, the expression of VHH-SAN1 also yielded the unexpected formation of VHH-SAN1-Nup120 puncta away from the NE. We hypothesize that this nanobody may weaken the affinity of the Y complex to the NPC assembly. These puncta could represent Y complexes that are slower to incorporate into the NPC assembly or potentially dissociated Y complexes from assembled NPCs. In either case, this is only a modest disruption, as the cells have no obvious growth defect.

Both Nup133 nanobodies (VHH-SAN4 and 5) were unable to localize to the NE in yeast cells (Fig. 6b). These nanobodies are most likely blocked from binding in the assembly by the Arf-GAP1 lipid packing sensing (ALPS) motif50 (Fig. 7b, d). Nup133 is thought to be anchored to the NE by its ALPS motif on its N-terminal β-propeller domain51,52, which is on the same face of Nup133 as the epitopes for VHH-SAN4 and 520. This suggests that the ALPS interaction with the membrane outcompetes the

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**Fig. 5 Structure of Nic96-VHH-SAN12 highlights the flexibility of the ACE1 fold.** a The structure of Nic96-VHH-SAN12. Nic96 is shown in gradient color from white to dark blue and VHH-SAN12 is shown in light blue. Helices are labeled on Nic96, along with the complementarity determining region (CDR) loops on VHH-SAN12. Boundaries of the ancestral cotamer element (ACE1) fold modules are indicated by the dashed lines. b Superposition of Nic96 with the previously solved structure (PDB: 2QX537) (shown in green). The alignment was done only in the tail module of the protein. c Superposition of the Nic96 crown module between the two structures. This structure of Nic96, shown in dark blue and the previous structure, shown in green.
binding of the nanobodies. While VHH-SAN4 has both fast on and off kinetics and the weakest binding affinity (230 nM), VHH-SAN5 binds Nup133 tightly in vitro (10 nM). This implies that the membrane interaction of the ALPS motif must have an even higher affinity or the epitope for VHH-SAN4 and 5 on Nup133 is blocked by the membrane very soon after new copies of Nup133 are synthesized, since the nanobodies are constitutively expressed.

Similar to VHH-SAN1, the Nup145C-specific VHH-SAN6 redistributes some Nup120 (and potentially Y complex) into cytoplasmic puncta, but does so to a lesser extent, along with its colocalization on the NE (Fig. 6b). Like VHH-SAN1 expression, the presence of these puncta has no effect on the fitness of the strain, so the pool of Y complex present on the NE must still be sufficient for proper cellular function. We cannot exclude at present that the expression of nanobodies like VHH-SAN1 might...
exert more subtle effects, for example by affecting the extent or rate of nuclear import/export of select cargoes. The Sec13 nanobody, VHH-SAN7, is the only nanobody in the library that decreases fitness as a mKate2 fusion, but not when expressed alone (Fig. 6a). From our fluorescence localization data, VHH-SAN7 exists mostly in cytoplasmic puncta, potentially due to the fact that Sec13 is present not only in the NPC, but also the COPII vesicle coat and SEA complex47,48. We hypothesize that VHH-SAN7 binds the copies of Sec13 outside of the NPC present in the cytoplasm, possibly by interacting with these copies of Sec13 more quickly after translation. Although we know VHH-SAN7 can bind Sec13 when assembled into the Y complex hub in vitro, it is also possible that its binding site is occluded in the context of the assembled NPC (Supplementary Fig. 1).

The Nup84 specific nanobodies (VHH-SAN8 and 9) behave differently due to binding opposite faces of Nup84. VHH-SAN8 binds the top surface of Nup84, away from the NE (Fig. 7b, d). This most likely explains why VHH-SAN8 colocalizes strongly with Nup120-GFP and causes no fitness defects. On the other hand, the expression of VHH-SAN9 has a peculiar effect on the localization of Nup120-GFP. Many cells showed diffuse fluorescence for VHH-SAN9, but in the general curvature of the NE. In many cases, we observed similar diffuse NE fluorescence of Nup120-GFP on part of the NE, but crisp NE rim fluorescence on the part of the nucleus where VHH-SAN9 was not present. VHH-SAN9 binds the side of Nup84 adjacent to the NE (Fig. 7b, d). It is possible that the presence of VHH-SAN9 disrupts the Y complex assembly on the NE, prying the Y complex away from the membrane. Interestingly, this occurs on only some of the NPCs within the same cell. Further investigation into the state of these NPCs and the NE is ongoing.

Both Nup120 specific nanobodies colocalize well with Nup120-GFP. The expression of VHH-SAN10 has little effect on the distribution of rim fluorescence of Nup120-GFP. Residues 197–216 are hypothesized to also be an ALPS motif and the overlapping residues 187–203 are required for VHH-SAN10 binding52. Docking of the Y complex into the cryo-ET map of the scNPC suggests this face of Nup120 to be positioned adjacent to the membrane24 (Fig. 7b, d). If Nup120 does bind the membrane at this loop, the nanobody likely outcompetes the affinity of Nup120 for the membrane. This would also suggest that the membrane-attachment by Nup120 may not be critical for NPC assembly and function. VHH-SAN11 had a more pronounced effect on the localization of Nup120-GFP. Some protrusions emanating from the NE were observed to have both Nup120 and VHH-SAN11, along with some foci in the cytoplasm.

VHH-SAN12 binds Nic96 between its trunk and tail modules. The interaction between the CDR loops of VHH-SAN12 and these trunk-tail interface helices maintains Nic96 in a different conformation than previously observed by X-ray crystallography45,46.
Most of the VHH-SAN12-mKate2 fusion enriched at the NE along with Nup120-GFP. However, VHH-SAN12 formed foci, both on the NE and in the cytoplasm and within the nucleus, while Nup120-GFP showed an even distribution on the NE. There are two possible explanations that can account for these foci. Either VHH-SAN12-mKate2 expression was limiting or not every NPC displays Nic96 in a conformation or conformation accessible for VHH-SAN12 binding. The concept of NPC heterogeneity has been observed and discussed in the literature, both in terms of its composition and in size52–53. Whether this interesting fluorescence is indeed due to NPC heterogeneity within the same cell is also under ongoing investigation.

The nanobody suite describes here provides a set of tools for studying the NPC assembly both in vitro and in vivo. In vitro, the nanobodies bind tightly to their targets and have enabled structural analysis of multiple nups at higher resolution than reported earlier or that evaded previous attempts by X-ray crystallography altogether29. In vivo, many nanobodies co-localize with the Y complex and therefore can be used as cellular tools in future studies on the NPC. For example, the nanobody library could enable subunit identification in future cryo-ET studies. In individual cases, such as VHH-SAN9 and VHH-SAN12, they have also provided questions for further study on NPC assembly and heterogeneity. Along with ref. 20, we also detailed the library’s breadth in antigen recognition, owing to its wide diversity in both CDR sequence and length. Overall, our data highlight the exciting potential of this nanobody library to be used as tools for both in vitro and in vivo studies of the NPC and pave the way for future explorations of NPC assembly and composition in S. cerevisiae.

**Methods**

**Construct generation.** All Nups (Nup120, Nup85, Seh1, Nup145, Sec13, Nup133, Nic96) were cloned from S. cerevisiae and expressed recombinantly in E. coli. Expression and purification were done as previously described59. The animal was purchased locally, maintained in the pasture, and immunized following a protocol authorized by the Tufts University Cummings Veterinary School Institutional Animal Care and Use Committee (IACUC). The animal was immunized against recombinantly expressed full-length Y complex (Nup120-Nup85-481) followed by phage precipitation from the resulting supernatant with 1% PEG-6000, 500 mM NaCl at 4 °C, and resuspended in PBS.

**Selection of VHHS by phage display.** VHHS were selected by panning against Nup120-1037-Nup145C34-712-Sec13, Nup85-757, Nup120, Nup145C-712-Sec13, Sec13-Nup145C34, Nup85-Nup145C34-Seh1, and Nup85-757 expression vectors were transfected into E. coli LOBSTR-RII (DE3)58 (Kerafast) cells and protein production was induced with 0.2 mM IPTG at 18 °C for 12–14 h. Cells were collected by centrifugation at 10,000 x g, resuspended in lysis buffer (50 mM potassium phosphate pH 8.0, 500 mM NaCl, 30–40 mM imidazole, 3 mM β-mercaptoethanol (BME), 1 mM PMSF, and a high-pressure cell homogenizer (Microfluidics L20)). The lysate was cleared by centrifugation at 12,500 x g for 25 min. The soluble fraction was incubated with Ni Sepharose 6 Fast Flow beads (GE Healthcare) for 30 min on ice. After washing the beads, the lysate with the beads were eluted, concentrated and loaded onto a Superdex S200 16/60 column (GE Healthcare) in buffer. Fractions containing all five proteins were pooled and concentrated. For all VHH complexes, the proteins were incubated for 30 min on ice. The incubated mixtures were then run on a Superdex S75 or Superose 10/300 column pre-equilibrated in GF buffer. Fractions containing the complexes were pooled and concentrated. To assemble the Y complex, we first mix 1.5x molar excess of Nup85-757 with Nup120-1037-Nup145C34-712-Sec13. After incubation for 30 min on ice, the complex was run on a Superose 26/10 300/10 column in GF buffer. Fractions containing all five Y complex components were pooled and concentrated.

**VHH library and M13 phage generation.** Alpaca immunization and library generation were done as previously described59. The animal was purchased locally, maintained in the pasture, and immunized following a protocol authorized by the Tufts University Cummings Veterinary School Institutional Animal Care and Use Committee (IACUC). The animal was immunized against recombinantly expressed full-length Y complex (Nup120-Nup85-481) followed by phage precipitation from the resulting supernatant with 1% PEG-6000, 500 mM NaCl at 4 °C, and resuspended in PBS.

**Protein expression and purification.** Nup120-1037-Nup145C34-712-Sec13, Nup85-757, Nup120, Nup145C-712-Sec13, Sec13-Nup145C34, Nup85-Nup145C34-Seh1, and Nup85-757 expression vectors were transfected into E. coli LOBSTR-RII (DE3)58 (Kerafast) cells and protein production was induced with 0.2 mM IPTG at 18 °C for 12–14 h. Cells were collected by centrifugation at 10,000 x g, resuspended in lysis buffer (50 mM potassium phosphate pH 8.0, 500 mM NaCl, 30–40 mM imidazole, 3 mM β-mercaptoethanol (BME), 1 mM PMSF, and a high-pressure cell homogenizer (Microfluidics L20)). The lysate was cleared by centrifugation at 12,500 x g for 25 min. The soluble fraction was incubated with Ni Sepharose 6 Fast Flow beads (GE Healthcare) for 30 min on ice. After washing the beads, the lysate with the beads were eluted, concentrated and loaded onto a Superdex S200 16/60 column (GE Healthcare) in buffer. Fractions containing all five proteins were pooled and concentrated.

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biotinylated, C-terminally Avi-tagged nanobody ligands to between 0.2–0.5 nm over 40–60 s. After dipping the coated biosensor tip in BLI buffer for 1 min, association was measured by analysis over 1–80 min. Dissociation was measured in BLI buffer for 1–220 min. All binding sensorgrams were recorded on a ForteBio OctetRED96 instrument. All fits were done using global, 1:1 kinetic binding parameters using the Octet data analysis software.

**Protein crystalization.** Initial hits of Nic96186–839-VHH-SAN12 were obtained at 18 °C in 1 day in a 96-well sitting drop tray with a reservoir containing 8% (v/v) PEG 8,000 and 0.1 M tri-sodium citrate pH 3.0 (Protein Complex suite, Qiagen). Hanging drops of 1 μl protein at 6 mg/ml and 1 μl of precipitant (7–10% (v/v) PEG 8000 and 0.1 M tri-sodium citrate pH 5.0) incubated at 18 °C produced diffraction quality rod-shaped crystals in 3 days. Crystals were transferred into a cryo-protectant solution containing the crystallization condition with 15% (v/v) glycerol and cryo-cooled in liquid nitrogen.

Initial hits of Nup1201-757-VHH-SAN10/11 were obtained at 18 °C in 3 days in a 96-well sitting drop tray with a reservoir containing 20% (v/v) PEG 8000, 0.2 M magnesium chloride, 0.1 M Tris/HCl pH 8.5 (JCSG + suite, Qiagen). Hanging drops of 0.1 μl protein at 2 mg/ml and 1 μl of precipitant (19% (v/v) PEG 8000, 0.1 M magnesium chloride, 0.1 M Tris/HCl pH 8.5) incubated at 18 °C yielded large rod-shaped crystals in 4 days. Crystals were transferred into a cryo-protectant solution containing the crystallization condition with 20% (v/v) glycerol and cryo-cooled in liquid nitrogen.

**Structure determination.** Data collection was performed at the advanced photon source end station 24-IDC. All data processing steps were carried out with programs provided through SQRgrid.7 Data reduction was performed using HKL2000.5 Statistical parameters of data collection and refinement are all given in Table 2. All manual model building steps were carried out with COOT and phenix.39 refinement was used for iterative refinement. Structure figures were created in PyMOL (Schrodinger LLC).

The structure of Nic96186–839-VHH-SAN12 was solved by MR using Phaser-MR in PHENIX. A solution was found by searching with the previously determined 536–1162 VHH-SAN12 structure from the model due to poor density. Secondary structure restraints were used throughout refinement. The two nanobodies, density only for VHH-SAN12, was present on the map. The structure of Nup85183–757–Seh1-VHH-SAN2 was solved by MR using Phaser-MR in PHENIX. A two-part MR solution was obtained by sequentially searching with models of Nic96 and VHH-SAN12. For Nic96, we used the previously solved structure from the model due to poor density. Secondary structure restraints were used throughout refinement. The two nanobodies, density only for VHH-SAN12, was present on the map.

**Fluorescence microscopy.** Strains were grown overnight in SD-Ura media (CSM-Ura (Sunrise Science), Yeast nitrogen base with ammonium sulfate, 2% (v/v) glucose) at 30 °C, followed by 20-fold dilution into fresh SC-Ura media. After growth for 4–5 h at 30 °C to OD600 ~0.5, cells applied to a thin SD-Ura agar pad on a standard microscopy slide and imaged live on a DeltaVision Elite Widefield Deconvolution Microscope (GE Healthcare) using a 100% oil immersion objective with an sCMOS camera (Teledyne Photometrics). Images were processed and analyzed in ImageJ.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Coordinates and structure factors have been deposited in the Protein Data Bank under PDB accession codes 6X06 (Nic96186–839-VHH-SAN10/11), 6207 (Nic96186–839-VHH-SAN12), 6X08 (Nup85183–757–Seh1-VHH-SAN2). The cryo-ET map used for docking of the Y complex and nanobodies is described elsewhere118 and available from the Electron Microscopy Data Bank (EMDB) under accession number EMD-10198.

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**Table 2 Data collection and refinement statistics.**

| Protein              | Nic96186–839–VHH-SAN12 | Nup1201–757–VHH-SAN10/11 | Nup85183–757–Seh1–VHH-SAN2 |
|----------------------|------------------------|--------------------------|---------------------------|
| PDB code             | 6X07                   | 6X06                     | 6X08                      |
| Organism             | S. cerevisiae, V. pacos |                          |                           |
| Data collection      |                         |                          |                           |
| Space group          | P 2, Z 2, Z 2         | P 6 1                    | P 6 2, Z 2                |
| Cell dimensions      | a, b, c (Å)            | a, b, c (Å)              | a, b, c (Å)               |
|                      | 48.1, 49.9             | 90, 90, 90              | 90, 90, 120              |
|                        | 79.5, 283.3            | 93.2, 78.1              | 234.0, 139.4             |
|                        | 90, 90, 90             | 90, 120, 120            | 90, 90, 120              |
| Resolution (Å)       | 60.9–2.1               | 82.7–4.3                | 117.0–4.2                |
| R_p.i.m. (%)         | (2.2–2.1) a            | (4.4–4.2) a             | (4.4–4.2) a              |
| R_free (%)           | 3.8 (67.7)             | 5.2 (51.2)              | 5.6 (66.5)               |
| Sigma (Å)            | 23.9 (1.2)             | 18.7 (1.4)              | 19.1 (1.3)               |
| C_Cry (Å)            | 1 (0.69)               | 0.94 (0.52)             | 0.98 (0.64)              |
| Completeness (%)     | 99.4 (98.9)            | 99.5 (99.2)             | 99.9 (99.9)              |
| Redundancy (%)       | 7.2 (6.9)              | 7.3 (7.2)               | 37.9 (39.9)              |
| R factor (Å2)        | 60.9–2.1               | 82.7–4.3                | 117.0–4.2                |
| Resolution range (Å) | 64.310                 | 11.641                  | 16.995                    |
| No. reflections      | 16.2 €/P                | 33.6/35.9               | 31.3/34.7               |
| No. atoms            | 5,786                  | 3818                    | 6890                     |
| Protein              | 140                    | 0                       | 0                        |
| Water                | 8 factors (Å2)         | 88.6                    | 241.0                    |
|                      | 69.4                   | 0.011                   | 0.002                    |
| r.m.s. deviations    | Bond length (Å)        | 1.101                   | 0.605                    | 0.949

Values in parenthesis are for the highest-resolution shell (10% of the data). One crystal was used for each dataset.
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Author contributions
S.A.N. and T.U.S. designed the study. S.A.N. performed the experiments. K.A. solved the structure of Nic96186-839-VHH-SAN12 and K.E.K. solved the structure of Nup1201–757-VHH-SAN10/11. J.R.I. and H.P. conducted the alpaca immunization experiments and generation of the phagemid library. S.A.N. and T.U.S. interpreted the results and wrote the manuscript with input from K.A., K.E.K., and H.P.

Competing interests
The authors declare no competing interests.

Additional information
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