N-terminal acetylation is one of the most common protein modifications in eukaryotes and is carried out by N-terminal acetyltransferases (NATs). It plays important roles in protein homeostasis, localization, and interactions and is linked to various human diseases. NatB, one of the major co-translationally active NATs, is composed of the catalytic subunit Naa20 and the auxiliary subunit Naa25, and acetylates about 20% of the proteome. Here we show that NatB substrate specificity and catalytic mechanism are conserved among eukaryotes, and that Naa20 alone is able to acetylate NatB substrates in vitro. We show that Naa25 increases the Naa20 substrate affinity, and identify residues important for peptide binding and acetylation activity. We present the first Naa20 crystal structure in complex with the competitive inhibitor CoA-Ac-MDEL. Our findings demonstrate how Naa20 binds its substrates in the absence of Naa25 and support prospective endeavors to derive specific NAT inhibitors for drug development.
α-acetylation is the most common protein modification in eukaryotes. Approximately 60% of all soluble proteins in yeast, more than 70% in plants, and 80–90% in humans are N-terminally acetylated. This modification is involved in many cellular processes affecting the stability, folding and degradation of proteins, protein interactions, subcellular localization and it is linked with several human diseases like cancer, Parkinson or Huntington disease. During N-terminal acetylation, an acetyl group is transferred from acetyl coenzyme A (AcCoA) to the α-amino group of a polypeptide. This modification is mostly carried out co-translationally by Nα-acetyltransferase complexes (NATs), which differ in subunit composition and substrate specificity, but comprise at least one catalytic subunit. Eight different eukaryotic NATs have been identified so far (NatA to NatH). The NatB-complex, one of the major NATs, is composed of two subunits, the catalytic subunit Naa20 and the auxiliary subunit Naa25 (formerly known as Nat3 and Mdm20, respectively). Both subunits are conserved within eukaryotic model organisms and the NatB complex is found associated with the ribosome. Deletion mutants of Naa25 and Naa20 in Saccharomyces cerevisiae show a slow growth phenotype, are unable to form actin cables, have a defect in vacuolar and mitochondrial inheritance and are sensitive to DNA damage causing agents. Recently, ScNaa20 dependent acetylation was suggested to have a protective function in regard to protein degradation and a role in protein synthesis. An involvement of the NatB complex in vacuolar protein sorting and cell wall maintenance, as well as in influencing the shutoff activity of influenza A virus was also suggested. Additionally, NatB is involved in the regulation of plant development, abiotic stress response and is linked to the microRNA pathway and in human cell proliferation, cell survival and liver cancer progression. noteworthy, as NatB subunits exhibit partly divergent phenotypes, it was speculated that both subunits may have functions independent from each other. Naa25 was found to not always coexpress with Naa20 in mouse neurons. Importantly, Naa25 was shown to be essential for the activity of Naa20 and localizes in the cytoplasm, while Naa20 is found in both, the nucleus and cytoplasm independent of Naa25.

NatB acts on substrates presenting N-termini with the initial methionine, which is retained and directly acetylated, followed by an acidic residue (MD-, ME-, MN- or MQ)·. Recently, crystal structures of Candida albicans NatB in complex with the bisubstrate inhibitor CoA-Ac-MDSEVA and in the free state were reported. They show that Naa20 adopts the canonical Gcn5-related N-acetyltransferase (GNAT) fold and accommodates a peptide at its substrate binding pocket. Naa25 forms a horse-shoe-like structure holding Naa20, and overall the GaNatB structures resemble the human NatB cryo-EM structure visualizing the high evolutionary conservation. However, while for the isolated catalytic subunits Naa10 and Naa50 crystal structures were reported, structural information on Naa20 were not available so far.

In order to dissect the molecular mechanism of NatB and its use as a potential therapeutic target, we studied Chaetomium thermophilum NatB complex and its individual subunits. Our results show that CnNaa20 is active towards a canonical NatB substrate without CnNaa25 in vitro, however less efficient than in NatB. We designed, synthesized and characterized the NatB inhibitor CoA-Ac-MDEL and solved the crystal structure of the catalytic subunit Naa20 in complex with this inhibitor. The structure reveals the basis of CnNaa20 substrate binding and activity towards the MDEL peptide.

**Results**

CnNaa20 binds with high affinity to CnNaa25. To functionally and structurally characterize the NatB complex and the subunits Naa20 and Naa25, we used the conserved orthologous proteins of the thermophilic model organism Chaetomium thermophilum (Ct) to benefit from their often superior properties in biochemical and structural studies. A BLAST search using the Candida albicans (Ca) NAA20 and NAA25 sequences as query revealed two candidates with 42% and 20% amino acids sequence identity, respectively (Supplementary Fig. 1). Next, full length CnNaa25, CnNaa20 and a truncated CnNaa20 variant were cloned and expressed in E. coli. The CnNaa20 variant was based on secondary structure prediction and comprises the predicted GNAT-fold. The auxiliary subunit CnNaa25 and the catalytic subunit CnNaa20 were individually expressed and purified to homogeneity (Fig. 1a, b). Co-expression of CnNaa25 and CnNaa20 led to the formation of the NatB complex, which could also be purified to homogeneity (Fig. 1c). In order to characterize the NatB complex, size exclusion chromatography coupled to multi angle light scattering (SEC-MALS) was carried out. The individual subunits CnNaa25 and CnNaa20 and NatB eluted as single symmetric peaks and analyses of the molecular mass showed that CnNaa20 and CnNaa25 are monomers in solution (Fig. 1a, b). A molecular mass of 136.5 kDa determined for NatB confirms a 1:1 stoichiometry of its subunits (Fig. 1c). To further characterize NatB complex formation, we performed isothermal titration calorimetry experiments. Titration of CnNaa25 into CnNaa20 was endothermic (ΔH = 5.4 kcal/mol) and resulted in the formation of a stable complex with a dissociation constant Kd of 17.8 ± 11.9 nM and a molar ratio of one (Fig. 1d).

As NatB acts co-translationally at the ribosome, we wanted to address ribosome binding of CnNaa20 and CnNatB. Electrophoretic mobility shift assays (EMSA) were performed to analyze CnNaa20 or CnNatB binding to RNA as indicator for putative ribosome interaction. CnNaa20 and CnNatB were incubated with a C. thermophilum expansion segment 27 RNA (GES27) fragment. ES27 was shown to be involved in the ribosome binding of the ribosome-associated factors NatA, Arx1, and Ebp36–38. A shift of the RNA band was observed upon addition of CnNatB but not for CnNaa20, indicating that CnNatB but not CnNaa20 alone can bind to GES27 (Fig. 1e). To analyze the specificity of the interaction, hammerhead ribozyme RNA was used as control (Supplementary Fig. 2). No RNA binding for CnNaa20 could be detected, while CnNatB binding to hammerhead RNA was observed, showing that CnNatB binds nonspecifically to RNA. This suggests that Naa20 does not associate with the ribosome on its own, but only when in complex with Naa25.

CnNatB acetylates specifically the MDEL peptide. We then investigated CnNatB substrate specificity and enzymatic activity by in vitro acetylation assays. Canonical NatA (SESS)3, Naa80/Naa10 (EEEI)39,40, NatB (MDEL)13 and NatC/E/F (MVNALE and MLGTE)3 substrates were tested. CnNatB acetylates only the MDEL peptide, highlighting that NatB specificity is conserved (Fig. 2a). The MDEL peptide was then used to determine the NatB enzymatic parameters. CnNatB showed a Michaelis constant (Km) of 45.6 ± 4.8 μM for AcCoA and a turnover number (kcat) of 68.8 ± 2.0 min⁻¹ (Fig. 2b). These values are in good agreement with the values observed for CaNatB and AtNatB (Supplementary Fig. 3a)23,30. Based on these results, we designed and synthesized a bisubstrate analog, CoA-Ac-MDEL (Fig. 2c and Supplementary Fig. 3b). This bisubstrate is a potent competitive NatB inhibitor with a half-maximum inhibitor concentration (IC50) of 1.56 ± 0.24 μM and an inhibitor constant Ki of 0.41 ± 0.14 μM (Fig. 2d).
The potency of CoA-Ac-MDEL in inhibiting CtNatB is in the same range as CoA-Ac-MVNAL inhibiting NatF and CoA-SASEA inhibiting NatA (Fig. 2e)\textsuperscript{32,41}. Taken together, our results show that NatB substrate specificity is evolutionarily conserved, and indicate that all NATs bind their specific inhibitors with similar affinities.

CtNaa20 is active and specifically acetylates the NatB substrate MDEL. To further characterize CtNatB, we investigated the activity of the catalytic subunit CtNaa20 alone. In contrast to previous reports on NatB, CtNaa20 shows a clearly detectable and specific activity towards the MDEL peptide (Fig. 3a) with a $K_m$ value for AcCoA of 12.0 ± 1.0 μM and a $k_{cat}$ of 9.0 ± 0.2 min\(^{-1}\) (Supplementary Fig. 4a). These data show that the catalytic subunit alone is active, but less efficient than in complex with CtNatB. The bisubstrate analog CoA-Ac-MDEL is also a potent inhibitor of CtNaa20, with an IC\(_{50}\) of 6.5 ± 2.5 μM (Supplementary Fig. 4b). Compared to NatB the higher IC\(_{50}\) indicates a lower affinity of CtNaa20 to this inhibitor. The difference between the enzymatic activities of CtNaa20 alone and as part of NatB might be explained by different affinities for the MDEL substrate. To test this, we performed kinetic experiments with constant AcCoA,
but varying MDEL concentrations. CtNatB shows a \( K_m \) of 232 ± 28 μM for MDEL, which is significantly lower than the one of CtNaa20 alone (4.4 ± 0.9 mM), while the \( k_{cat} \) is in a similar range (Supplementary Fig. 4c, d). These data suggest that CtNaa25 increases the affinity of CtNaa20 for NatB substrates.

To further investigate the CtNatB and CtNaa20 ligand interactions, we performed nanoDSF based binding assays using CoA-Ac-MDEL and AcCoA. Binding of both ligands to CtNatB and CtNaa20 is indicated by protein stabilization (Fig. 3b, c). When AcCoA is added to CtNatB, a mild stabilizing effect was observed by an increase of the unfolding transition temperature from 58 °C to 62 °C (using a 1/64 ratio of protein/ligand). Upon addition of the bisubstrate analog, CtNatB melting temperatures increased drastically from 58 °C to 71 °C. This illustrates a major contribution of the NatB specific peptide to the stabilization of the protein, compared to AcCoA alone (Fig. 3b). Noteworthy, the bisubstrate has a stronger effect on CtNatB melting temperature increase than AcCoA. These data confirm that CtNaa20 alone is able to bind MDEL and support the observation that it can acetylate MDEL without CtNaa25 (Fig. 3c). As a control we used two similar bisubstrate analogs with different peptide moieties in the nanoDSF assay (Fig. 3d). CoA-Ac-SESS and CoA-Ac-MVNAL were described as NatA and NatF inhibitors, respectively. As they contain a CoA moiety, they exhibit a mild stabilizing effect on CtNatB, which is significantly lower than the effect of CoA-Ac-MDEL. The difference in stabilization highlights that MDEL binds to CtNaa20, but not the other peptides. Noteworthy, when only the MDEL peptide was added in a saturating amount to CtNatB or CtNaa20, no significant stabilizing effect was detected (Supplementary Fig. 4e). This is in accordance with the mechanism reported for Naa50, where AcCoA needs to bind before a substrate can bind. Taken together, our data clearly show that CtNaa20 is active towards a canonical NatB substrate without CtNaa25. However, CtNaa25 increases the CtNaa20 affinity for this substrate, allowing for a more efficient acetylation.

**CtNaa20 crystal structure in complex with CoA-Ac-MDEL.** So far, structural information on Naa20 in the absence of the adaptor subunit Naa25 has not been available. In order to characterize Naa20 on an atomic level, we crystallized CtNaa20 in complex with the bisubstrate analog CoA-Ac-MDEL. The structure was

![Fig. 2 CtNatB acetylation activity and inhibition. a Substrate specificity of CtNatB tested with five different peptides. SESS, EEEI, MDEL, MLGTE and MVNALE were previously identified as NatA, Naa10/Naa80, NatB and NatC/E/F substrates. b Michaelis–Menten curve of the CtNatB mediated acetylation of MDEL. c Structure of the bisubstrate analog CoA-Ac-MDEL. d Dose-response curve of the NatB mediated acetylation, inhibited by CoA-Ac-MDEL. e Inhibitor characteristics of CoA-Ac-MDEL compared to other bisubstrate analogs. All reactions were performed in triplicates and error bars represent the standard deviation.](https://doi.org/10.1038/s42003-020-01546-4)
solved in space group P 2₁ with two molecules per asymmetric unit. The initial phases were obtained by molecular replacement with the CaNaa20 structure part of the CaNatB complex (pdb: 5k18)30. The structure could be built at 1.57 Å resolution, revealing the expected GNAT-fold (Table 1; Fig. 4a). The high quality of the electron density map allowed building residues 2–190 together with one CoA-Ac-MDEL ligand (with the peptide part M1pD2pE3pL4p) for both protein chains. The root mean square deviation (rmsd) between the two protein molecules is 0.2 Å (for 190 Ca atoms), indicating a very low level of flexibility.

The CaNaa20 structure consists of 4 a-helices and 8 b-strands and the bisubstrate is bound in the known V-shaped binding groove typical for NATs30,33,41,42. The b8-strand is a short additional strand compared to other NATs and shows that CaNaa20 C-terminal residues fold back along the b6 strand (Fig. 4a). This interaction stabilizes the enzyme compared to a CaNatB complex, but also shows minor differences. The CaNaa20 structure explains the substrate specificity. The CaNaa20 structure demonstrates how the catalytic subunit binds a substrate peptide in the absence of CaNatB. The loops α1-α2 and β6-β7 and the elongated β3-β4 loop fold over the substrate peptide and contacts the α1-α2 loop, while the CaNatB β6-β7 loop turns away from the peptide (Fig. 4a, b). This results in a narrower peptide binding site in CaNaa20 compared to CaNatB. Taken together, we obtained a high-resolution crystal structure of CaNaa20 alone, which superimposes well with CaNaa20 as part of the CaNatB complex, but also shows minor differences.

**Figure 3** **CaNaa20 acetylation activity and CaNaa20/CaNatB-ligand interaction.** a) Substrate specificity of CaNaa20 tested with five different peptides. b) Melting temperatures of CaNatB in the presence of varying concentrations of AcCoA and CoA-Ac-MDEL. c) Melting temperatures of CaNaa20 in the presence of varying concentrations of AcCoA and CoA-Ac-MDEL. d) Melting temperatures of CaNatB and CaNaa20 in the presence of varying concentrations of CoA-Ac-MDEL, CoA-Ac-SESS, or CoA-Ac-MVNAL. All measurements were performed in triplicates and error bars represent the standard deviation.
Supplementary Fig. 5a). Noteworthy, this pocket is less hydrophobic compared to methionine pockets of other NATs, like Naa50 or Naa60, which also act on the initiator methionine.\(^33,41,45\) The M1p backbone carbonyl is bound by the hydroxyl group of Y145 and the D2p amide is coordinated by the backsidechain (Fig. 4c). The D2p backbone carbonyl binds to the Y27 sidechain and the amide of E3p hydrogen bonds to Y144 sidechain (Supplementary Fig. 5a). The E3p peptide sidechain hydrogen bonds to the G146 backbone (Supplementary Fig. 5a), but no sidechain specific protein-ligand interactions are found for E3p and L4p, highlighting that the substrate specificity is mainly determined by the first two positions. Noteworthy, a well-ordered water can be found in the active site, which may be involved in catalysis. This water is coordinated by the backbone of F118 and 181, the M1p amide and the D2p sidechain, (Supplementary Fig. 5b).

Besides, the electrostatic surface potential of CNaa20 reveals a positive area at the conserved AcCoA binding site, but no further exposed positive patches (Supplementary Fig. 5c), corroborating the lack of CNaa20 binding to RNAs (Fig. 1e and Supplementary Fig. 2). In summary, the structural data support the observation that CNaa20 acetylates canonical NatB substrates in vitro and show that H80 is important for specific peptide binding.

### Specific residues are crucial for CNatB activity
To further investigate the enzymatic mechanism of CNatB, we mutated residues in CNaa20 which are suggested to be important for acetylation efficiency.\(^30\) Single mutations in the CNaa20 substrate-binding pocket and active site (Y27A, H80A, H80Y, F118A, and Y145A) do not affect protein stability (Fig. 5a), but impair CNatB acetylation efficiency (Fig. 5b). When CNaa20 Y27 was replaced by F, which is the corresponding residue in CnNaa20 (Supplementary Fig. 1b), or F118 replaced by H, the corresponding residue in Naa10, Naa50 and Naa60 (Supplementary Fig. 6a), the catalytic efficiency of the resulting CNatB complex does not change (Fig. 5b). Noteworthy, the H80Y and F118H CNatB mutants and the corresponding double mutant, which were created to mimic the Naa10, Naa50 or Naa60 sequences at these positions (Supplementary Fig. 6a), are not sufficient to alter the substrate specificity of NatB to accept SELSS, EEEI or MVNAL peptides (Supplementary Fig. 6b). To test whether CNaa20 H80 is the major determinant for Naa20 and NatB substrate specificity (Fig. 4c), we created the Arabidopsis thaliana Naa60 Y115H mutant, to mimic the Naa20 sequence at the corresponding position (Supplementary Figs. 1b and 6a). This Naa60 mutant acetylated the NatB substrate MDEL in addition to its canonical substrates (Supplementary Fig. 6c), while the AtNaa60 wild-type is not active towards MDEL.\(^41\) This confirms that H80 is indeed a key residue for Naa20 substrate specificity.

The inactive CNaa20 mutants (Y27A, H80A, H80Y, F118A, and Y145A) were also tested for CoA-Ac-MDEL binding using nanoDSF (Supplementary Fig. 7). The stabilizing effect of CoA-Ac-MDEL is higher than that of AcCoA for the Y27A and F118H mutant, indicating that they still bind MDEL (Supplementary Fig. S7a, e). In contrast, the H80A, H80Y, F118A, and Y145A mutants are not able to bind MDEL (Supplementary Fig. 7b–f) explaining their acetylation deficiency. Comparison with CnNaa20 shows that the corresponding residues superimpose well (Fig. 5c), which implies that their role in peptide binding and substrate acetylation is conserved between Naa20 proteins. Taken together, our data show that CNaa20 alone is active in substrate acetylation and highlight the importance of specific residues for peptide binding and acetylation activity, and suggest that Naa25 binding does not induce conformational changes in Naa20.

### Discussion
The majority of the proteome is N-terminally acetylated with around 20% being acetylated by NatB in yeast, plants, and human.\(^46\) However, compared to the major NatA/NatE complexes, NatB has been less studied. For NatA and NatE, structures of the complexes\(^32,42,47\) and of the individual catalytic subunits Naa10 and Naa50\(^32,33\) were reported and analyzed in depth. Recently, the CnNatB crystal structure and the HisNatB cryo-EM structure were determined.\(^30,31\) This showed how Naa25 binds to Naa20, and provided the first molecular basis of NatB substrate specificity. However, the Naa20 subunit could not be purified alone in previous CnNatB and Arabidopsis thaliana (At) NatB studies.\(^23,30\) Therefore, to characterize NatB in more detail, we aimed to investigate CNaa20 structure and function in the absence of CNaa25.

Both CNatB subunits were expressed and purified independently to homogeneity, and were shown to be monomers in solution. A stable CNatB complex was formed with a 1:1 stoichiometry and a Kd of 17.8 ± 11.9 nM. Comparison with NatA binding to Naa50 (Kd = 46 ± 8.8 nM)\(^48\) shows that the binding affinities are in the same range. CNatB acetylates specifically the MDEL peptide with enzymatic parameters similar to the ones described for other organisms. This emphasizes a high degree of conservation of NatB substrate specificity and mode of action.\(^23,30,49\) Surprisingly, we also observed that CNaa20 alone specifically acetylates the MDEL peptide in vitro, but with a lower efficiency than CNatB. So far, Naa20 was considered to be inactive without Naa25 in vivo\(^15,16,24\) and the in vitro activity was not tested due to the lack of stable Naa20. In order to understand the seeming discrepancy between in vivo inactivity and the in vitro activity described in this study, we determined the

### Table 1 Data collection and refinement statistics (molecular replacement).

| Data collection | CnNaa20/CoA-Ac-MDEL |
|-----------------|---------------------|
| Space group     | P 1 2 1 |
| Cell dimensions | a, b, c (Å) | 46.2, 114.5, 47.4 |
| Resolution (Å) | 47.42-1.57 (1.60-1.57) |
| Rmerge          | 0.087 (1.306) |
| I/σ(I)          | 8.6 (1.4) |
| Completeness (%)| 98.7 (98.4) |
| Redundancy (%)  | 6.6 (6.9) |
| Refinement      | 47.42-1.57 (1.60-1.57) |
| No. of reflections | 65018 (6699) |
| Rwork/Rfree (%) | 17.1/20.6 |
| No. of atoms    | Protein | 3196 |
|                 | Ligand | 102 |
|                 | Water | 577 |
| B-factors (Å²)  | Protein | 29.1 |
|                 | Ligands | 28.0 |
|                 | Water | 40.6 |
| R.m.s deviations | Bond lengths (Å) | 0.011 |
|                 | Bond angles (°) | 1.035 |

*Values in parenthesis are for the highest-resolution shell.

The structure was determined from one crystal.

}\(^a\) The inactive CNaa20 mutants (Y27A, H80A, H80Y, F118A, and Y145A) were also tested for CoA-Ac-MDEL binding using nanoDSF (Supplementary Fig. 7). The stabilizing effect of CoA-Ac-MDEL is higher than that of AcCoA for the Y27A and F118H mutant, indicating that they still bind MDEL (Supplementary Fig. S7a, e). In contrast, the H80A, H80Y, F118A, and Y145A mutants are not able to bind MDEL (Supplementary Fig. 7b–f) explaining their acetylation deficiency. Comparison with CnNaa20 shows that the corresponding residues superimpose well (Fig. 5c), which implies that their role in peptide binding and substrate acetylation is conserved between Naa20 proteins. Taken together, our data show that CNaa20 alone is active in substrate acetylation and highlight the importance of specific residues for peptide binding and acetylation activity, and suggest that Naa25 binding does not induce conformational changes in Naa20.

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CtNaa20 RNA binding capability and analyzed the CtNaa20 electrostatic surface potential. CtNaa20 does not bind RNA and does not present exposed positive patches that would allow for a direct ribosome interaction (Fig. 1e and Supplementary Fig. 5c). Therefore, Naa20 alone most likely does not bind to the ribosome and might have limited access to its substrates, which could explain the in vivo inactivity of Naa20. For comparison, Naa10 does not expose positive patches and is not involved in NatE-ribosome interactions36. Noteworthy, Naa20 is also found in the nucleus as a single subunit13 and thereby might acetylate a subset of presumed NatB substrates post-translationally. Interestingly, NatB-type substrates are found overrepresented in the nucleus compared to the whole worm lysate in C. elegans and a higher acetylation rate is observed in the nuclear fraction12.

The observed differences in CtNatB and CtNaa20 acetylation efficiency are reminiscent of differences in the activity towards the MLGP peptide between human Naa50 alone and as part of the human NatE complex48. In this case, HsNaa50 in complex with HsNatA (forming HsNatE) increased its affinity for the peptide, which lead to a more efficient acetylation. Similarly, we showed
by enzymatic measurements that one function of Naa25 is to increase the affinity of Naa20 for NatB substrates. Probably, this is due to a stabilizing effect of CtNaa25 on CtNaa20, which leads to a more efficient acetylation.

Based on our results, showing that CtNaa20 specifically acetylates the MDEL peptide, we designed and synthesized the CoA-Ac-MDEL bisubstrate analog. This ligand was used for co-crystallization with CtNaa20 and structure determination at 1.57 Å resolution. The structure with the CoA-Ac-MDEL ligand allowed understanding the observed substrate specificity for the MDEL peptide. The M1p sidechain of the ligand is located in a wide pocket and importantly the D2p sidechain is coordinated by the H80 sidechain. Compared to other catalytic subunits acting on the initiator methionine, like Naa50 or Naa60, this specific sidechain interaction with the substrate residue in position two is a unique feature of Naa20 and of NatB. The responsible histidine sidechain is conserved in all Naa20 subunits (Supplementary Fig. 1b) and explains the specificity for acidic residues at substrate position two. Naa30, Naa50, or Naa60 have a tyrosine at this position (Supplementary Fig. 6a). Nevertheless, a single H80Y mutation in CtNaa20 was not sufficient to change the substrate specificity to NatC/E/F like substrates. The CtNaa20 methionine binding pocket is more hydrophilic compared to Naa50 and Naa60, consistent with Naa20 specificity for polar residues in position two. The substrate peptide residues E3p and L4p are not involved in specific protein-ligand interactions and may play only a minor role in substrate recognition. Accordingly, the third and fourth position of NatB substrates was found to be highly variable.

When compared with the CaNaa20 subunit in complex with CaNaa25, CtNaa20 alone superimposes very well and only differs in several loop regions. The CtNaa20 β3-β4 loop is longer compared to Caα, and the β6-β7 loop folds over the MDEL peptide and is in closer contact to loop α1-α2 than in Caα. The high similarity...
of both structures suggests that Naa25 does not induce major rearrangements in Naa20 and that the mechanism of substrate binding and acetylation is very similar between NatB and the isolated Naa20 subunit. Similarly, binding of CaNaa20 seems not to induce conformational changes in the CaNaa20 subunit39. Here, NatB differs distinctly from NatA, as the peptide-binding pocket of Naa20 remains unchanged upon NatB complex formation and Naa20 is catalytically active on its own. Formation of NatA induces rearrangements of catalytically important residues in Naa10 and alters its substrate specificity as Naa10 alone is not active towards NatA substrates32. The CaNaa20/CaA-Ac-MDEL structure supports the observation that CaNaa20 is active towards canonical NatB substrates in vitro. Nevertheless, we cannot exclude that CaNaa20 is also active towards other substrates in the absence of CaNaa25.

Our structure unravels the Naa20 peptide binding mode, but does not allow to deduce an exact catalytic mechanism. In general, NATs were shown to use a base-mediated mechanism. Naa10 uses a distinct glutamate as general base and the corresponding E25 of CaNaa20 is conserved in Naa20 proteins (Supplementary Figs. 1b and 6a)32. However, in the CaNaa20 structure, E25 is not positioned in a way to serve as a base and the corresponding E25A mutation in the CaNAT complex even increased the catalytic efficiency30. Naa50 and Naa60 were shown to use a dual-base mechanism with conserved tyrosine and histidine residues and a well-ordered water molecule33,41. A well-ordered water was also found in the active site of the CaNaa20 structure. This water is likely to be involved in the catalysis, however, a basic residue is not involved in the coordination of the water and therefore a definite catalytic mechanism cannot be deduced. In CaNaa20, H80 corresponds to the catalytically important tyrosine of Naa50/60 and is important for binding D24, but H80 cannot additionally hydrogen bond to the active site water or the M1p amide. The CaNaa20 residue corresponding to the catalytically important histidine of Naa50/60 is F118, which again is crucial for peptide binding and involved in coordinating the active site water, but is not a basic residue and therefore cannot be involved in proton transfer. Additionally, the F118H mutant showed no change in acetylation efficiency, whereas the corresponding H154F mutation in Naa60 impaired activity41. However, one has to consider that the bisubstrate analogs used here and in previous structures do not reflect the accurate transition state geometry of N-terminal acetylation, which proceeds via a tetrahedral conformation of the transferred acetyl-group carbon. This different geometry may hinder the identification of the CaNaa20 catalytic base, or the exact role of the active site water. Nevertheless, the CoA-Ac-MDEL bisubstrate analog is a potent competitive NatB inhibitor and a useful tool to understand the binding mechanism, structural features, and function of NATs. As NAT dysregulation is linked to multiple human diseases9, it is momentous to design NAT inhibitors for therapeutic purposes, based on substrate specificities and available structures, and to design ways to synthesize bio-available inhibitors in the future. NatB is an interesting therapeutic target because its depletion leads to the most severe phenotypes among all NATs30.

Taken together, our data contribute to a detailed understanding of NAT structures and functions, by providing the first Naa20 crystal structure. We show how Naa20 binds and specifically acetylates its substrates, which indicates that Naa20 may have a function without Naa25. Therefore, our study provides the structural and mechanistic framework to fully integrate NatB into the landscape of N-terminal protein acetylation.

**Methods**

**Structures of Naa20 constructs.** CaNaa25 was amplified by PCR from cDNA and an internal NcoI-site was abolished by introducing a silent mutation in the forward primer. The resulting fragment was digested with the NcoI and BamHI enzymes and ligated into the pETNHis-vector (G. Stier, BZH) to obtain the pETNHis: CaNaa25 construct with a TEV-site cleavable His-tag. CaNaa20 was amplified from cDNA by PCR and transferred into the TOPO-vector (Thermo Fischer). One unique MluI-cutting site was incorporated by site-directed mutagenesis. The C-terminal His-tag was introduced. The CaNaa20 fragment was ligated into the pET21d vector, leading to the pET21d::CaNaa20-His construct. Finally, the C-terminally truncated construct pET21d::CaNaa20Δ1-166-His was obtained by PCR using the full-length construct as template and cloning into the pET21d vector.

For the purifications of CaNaa20 subunit, the CaNAT complex or the complex with Naa20Δ1-166 point mutants, a three-step purification was performed. For 18 h, the pETNHis:CtNaa20 construct, was transformed into E. coli BL21 (DE3) E. coli strain (Novagen). Cells were grown at 18 °C in auto-induction-media, supplemented with chloramphenicol (34 μg ml⁻¹) and ampicillin (50 μg ml⁻¹). For purification, the cells were resuspended in lysis buffer (20 mM HEPEs pH 7.5, 200 mM NaCl, 40 mM Imidazole), supplemented with a protease inhibitor mix, lysed using a microfluidizer (M-110L, Microfluidics) and the lysate was cleared by centrifugation (50000 x g, 25 min, 4°C). For Ni-IMAC (immobilized metal affinity chromatography), the supernatants were loaded on a 1 ml HisTrap HP column (GE Healthcare). The tagged protein was eluted by adding 250 mM imidazole to the lysis buffer and further purified by size exclusion chromatography (SEC) using a S75 16/60 gel-filtration column (GE Healthcare) and buffer G (20 mM HEPEs pH 7.5, 200 mM NaCl).

For the purification of the CaNaa20 subunit, the CaNAT complex or the complex with Naa20Δ1-166 point mutants, a three-step purification was performed. For 18 h, the pETNHis:CtNaa20 construct, was transformed into E. coli BL21 (DE3) E. coli strain (Novagen). Cells were grown at 18 °C for 18 h in auto-induction-media, supplemented with chloramphenicol (34 μg ml⁻¹) and kanamycin (50 μg ml⁻¹) for the CaNaa25 expression and additionally with carbobenzoxy (50 μg ml⁻¹) for the different CaNAT complex species. For purification, the cells were resuspended in lysis buffer, supplemented with a protease inhibitor mix, lysed using a microfluidizer (M-110L, Microfluidics) and the lysate was cleared by centrifugation (50000 g, 25 min, 4°C). The proteins were purified with Ni-IMAC by loading the supernatant on two 1 ml HisTrap HP column (GE Healthcare). Elution was performed with 250 mM imidazole. Afterward, the samples were dialyzed against IEX buffer A (100 mM NaCl and 50 mM sodium citrate pH 5.5) and loaded on a 5 ml HiTrap SP column for cation exchange chromatography. The proteins were eluted using IEX buffer B (50 mM sodium citrate pH 5.5 and 1100 mM NaCl) by applying a step gradient of 18% NaCl.

**Cristallization of CaNaa20.** Crystallization was performed at 18 °C using the sitting drop vapor diffusion method. CaNaa20 was concentrated after gel-filtration to 20 mg/ml and mixed in a 1:3 molar ratio with CoA-Ac-MDEL and incubated on ice for 18 h. The crystallization drops contained 200 nl protein solution and 200 nl precipitant solution (15% (v/v) propanol, 0.2 M ammonium acetate, and 0.1 M TRIS pH 8.5). Crystals appeared after 3 days and were cryo-protected with 20% glycerol and flash-frozen in liquid nitrogen.

**Data collection and structure determination.** Data sets for the CaNaa20 crystals were collected at beamline P14 (DESY) at cryogenic temperature. The images were integrated with XDS53. Afterwards the images were scaled using AIMLESS52. The initial calculation showed that the crystals belonged to the space group I4122, with unit cell parameters a = b = 87.6 Å and c = 128 Å. Subsequently, the structure was solved by molecular replacement with PhaserMR39 implemented in the PHENIX package54. The CaNaa20 part of the CaNAT complex (96 pdb:5k18) was used as an initial search model. Finally, iterative model building and refinement were performed with Coot50 and Phenix.refine56. The CoA-Ac-MDEL ligand was parametrized with the PHENIX eLBOW module in AM1 QM model. Model quality was analyzed with MolProbity59. The crystallographic R-factor and the weighted R-factor were 21.5% and 26.3% respectively.

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**SEC-MALS analyses.** The CaNAT complex (0.12 mg) and its subunits CaNaa25 (0.13 mg) and CaNaa20 (0.1 mg) were successively injected onto a Superdex 200 10/300 gel-filtration column (GE Healthcare) in buffer G. The column was connected to a MALS system (Dawn Helesio II 8+ and Optilab T-Rex, Wyatt Technology). Data were analyzed using the Astra 6 software (Wyatt Technology).
Synthesis of CoA-Ac-MDEL inhibitor. For the synthesis of the bisubstrate analog CoA-Ac-MDEL, the Foyen et al. protocol was modified. MSPE peptide was synthesized with MultiSep Rsi peptide synthesizer (Initavis) on solid support. A leucine preloaded 2-chlorotriyl resin (50 µmol, 1 equiv.) was used with 9-Fluorenlymethoxycarbonyl (Fmoc)-amino acids (250 µmol, 5 equiv), HBTU (1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro-phosphate; 250 µmol, 5 equiv), HOBT (1-hydroxybenzotriazole; 0.2 mol/l) and DIPEA (Diisopropylcarbodiimide; 500 µmol, 10 equiv) in DMF (N,N-Dimethylformamide) doing double couplings for 40 min. Fmoc deprotection was carried out applying 40% piperidine in DMF for 3 min and then 20% piperidine in DMF for 14 min. 70 mg Bromoacetic acid (500 µmol, 10 equiv), dissolved in DMF and mixed with 155 µl 4 N HBr/500 µl 250 µmol, 5 equiv) in DMF was added to the peptide-linked resin. The suspension was shaken for 24 h at room temperature and then the resin was washed three times with DMF and DCM (Dichloromethane), respectively. The bromo-acetylated peptide was cleaved from the resin by gently shaking in a cleavage cocktail (Triisopropylsilane:Triisopropylsilane:water 1:1:2) for 3 h and was precipitated in 40 ml cold diethyl ether and dried under vacuum. The precipitate was dissolved in 2 ml water/acetonitrile mixture (80:20) and purified by reverse phase HPLC. The solvent was removed using a rotary evaporator revealing a white solid (3.5 mg, 5.6 µmol, 11.2% yield). Subsequently, the residue was evaporated to dryness by reverse phase HPLC (water/acetonitrile 10-30%) to a purity of >95%. The solvent was removed by lyophilization to give 0.9 mg of CoA-Ac-MDEL (0.7 µmol, 12.5% yield for CoA coupling; MALDI/TOF (pos): m/z calc. for C43H71N11O26P3S2+ found: 1314.32 [M + H]+; 1314.2).

CtNatb activity and CoA-Ac-MDEL inhibition assays. All enzymatic assays (the substrate specificity tests of CtNatb and CtNatb2, the Michaelis–Menten analysis of the CtNatb complex and the complex with the mutated catalytic subunits, the inhibitor assays to determine the Km of CoA-Ac-MDEL analog and the mode of inhibition) were performed using microplate assays described earlier and used as modified recently. For all assays a protein concentration of 500 nM was used. For all assays, either a constant peptide concentration of 1.5 mM or a constant AcCoA concentration of 370 µM with varying concentrations of 6–500 µM AcCoA or 39–2500 µM MDEL were used. Background control reactions were performed in the absence of the enzyme, or of the peptides and all reactions were performed in triplicates. Data were evaluated using the GraphPad Prism software.

ITC Measurements. ITC binding measurements between CtNatb25 and CtNatb25,106 were performed using a PEAC-ITC microcalorimeter (Malvern Instrument GmbH). Prior to the measurements, the protein samples were dialyzed against buffer G overnight. CtNatb106,25 concentrations of 25–35 µM in the cell were titrated with CtNatb25 concentrations of 250–400 µM in the syringe at 20 °C. The data were fitted and analyzed using a single-site binding model in the MicroCal PEAC-ITC analysis software. Measurements were performed in triplicates. In addition, buffer to buffer, buffer to CtNatb25,106 and CtNatb25 to ITC runs were performed as control reactions.

NanofDS Measurements. To determine melting temperatures Tm of different protein samples, nano differential scanning fluorimetry (nanoDSF) was used. Intrinsic tryptophan and tryptophan fluorescence at emission wavelengths of 330 nm and 350 nm were measured continuously applying a temperature gradient of 20–90 °C in the PerkinElmer FT.48 nanoDSF system. The Tm was calculated by the supplied software [NanoTemp Technologies GmbH]. To assess the stability of the different Ctnatb mutants, 1 mg/ml samples were measured in buffer G. The stability changes of Ctnatb, Ctnatb25 and its mutants upon addition of different ligands, were measured using 15–30 µM protein in buffer G, after incubation with a varying excess of AcCoA, CoA-Ac-MDEL, CoA-Ac-SESS, or CoA-Ac-MVNA for 10 min on ice.

Statistics and Reproducibility. All kinetic experiments were performed in triplicates. Error bars in figures represent the standard deviations. All nanofDSF assays were performed in triplicates or quadruplicates and error bars represent the standard deviations. Individual data points are depicted in all figures, apart for Fig. 7b. For Fig. 7b, kcat and Kd values of each mutant were determined in individuals Michaelis-Menten experiments in triplicates, which were further used to calculate the enzymatic efficiency, normalized to the wild-type efficiency. The data represent the mean values with standard deviations considering the propagation of uncertainty. The ITC measurements were performed in triplicates and Kd and ΔH represent the mean values with corresponding standard deviations.

Data availability
Coordinates and structure factors have been deposited at the Protein Data Bank under the accession code 6ZMP. All source data underlying graphs and charts are presented in Supplementary Data 1. Further data supporting the findings of this study are available from the corresponding author upon reasonable request.

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References
1. Arnesen, T. et al. Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc. Natl Acad. Sci. USA 103, 1097–1102 (2006).
2. Bienvenut, W. V. et al. Comparative large scale characterization of plant versus mammal proteins reveals similar and idiosyncratic N-α-acetylation features. Mol. Cell. Proteom. 11, M111.015131 (2012).
3. Van Damme, P. et al. The N-terminal acetylome of the human cell: toward a functional understanding of protein N-terminal acetylation. PLoS Biol. 9, e1001074 (2011).
4. Dörfel, M. J. & Lyon, G. J. The biological functions of Naal10—from amino-terminal acetylation to human disease. Gene 567, 103–131 (2015).
5. Starheim, K. K., Geraert, K. & Arnesen, T. Protein N-terminal acetyltransferases: where to start. Trends Biochem. Sci. 37, 152–161 (2012).
6. Gauthuis, M. et al. The yeast Na-acytetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides. Mol. Cell. Biol. 23, 7403–7414 (2003).
7. Arnesen, T. et al. Identification and characterization of the human ARD1–NATH protein acetyltransferase complex. Biochem. J. 386, 433–443 (2005).
8. Åknes, H., Ree, R & Arnesen, T. Co-translational, Post-translational, and Non-catalytic Roles of N-Terminal Acetyltransferases. Mol. Cell 73.6, 1097–1114 (2019).
9. Poleyvoda, B., Arnesen, T. & Sherman, F. A synopsis of eukaryotic Nα-terminal acetyltransferases: nomenclature, subunits and substrates. In BMC proceedings 3 S2 (BioMed Central, 2009).
10. Poleyvoda, B., Brown, S., Cardillo, T. S., Rigby, S. & Sherman, F. Yeast N-terminal acetyltransferases are associated with ribosomes. J. Cell. Biochem. 103, 492–508 (2008).
11. Gao, J. et al. N-terminal acetylation promotes synaptonemal complex B (hNatB): a complex important for cell-cycle progression. Genetics 161, 71–81 (2009).
12. Gao, J. et al. The human Nα-terminal acetyltransferase and of actin and tropomyosin. Proc. Natl Acad. Sci. USA 106, 8157–8162 (2009).
13. Singer, J. M., Hermann, G. J. & Shaw, J. M. Suppressors of mdm20 in yeast identify new aneles of ACT1 and TPM1 predicted to enhance actin-tropomyosin interactions. Genetics 156, 523–534 (2000).
14. Singer, J. M. & Shaw, J. M. Mdm20 protein functions with Nα-terminal acetyltransferase Tpm1 and regulates tropomyosin–actin interactions in budding yeast. Proc. Natl Acad. Sci. 100, 7644–7649 (2003).
15. Kats, I. et al. Mapping degradation signals and pathways in a eukaryotic N-terminome. Mol. Cell. Proteom. 7, 2251–2263 (2008).
16. Polevoda, B., Cardillo, T. S., Doyle, T. C., Bedi, G. S. & Sherman, F. Nat3p and ARD1 anchor to the ribosome and interacts with nascent polypeptides. Mol. Cell. Biol. 23, 7403–7414 (2003).
17. Kats, I. et al. Mapping degradation signals and pathways in a eukaryotic N-terminome. Mol. Cell. Proteom. 7, 2251–2263 (2008).
18. Nguyen, K. T., Kim, J.-M., Park, S.-E. & Hwang, C.-S. Synthetic lethal screen of yeast ribosomal proteins and its effect on protein synthesis. J. Proteom. 74, 431–441 (2011).

19. Kamita, M. et al. N-terminal methionine removal of mRNA during aminoacyl-tRNA biosynthesis. Structure 27, 1057–1070 (2019).

20. Lee, K.-E., Ahn, J.-Y., Kim, J.-M. & Hwang, C.-S. Synthetic lethal screen of Naa20, a catalytic subunit gene of NatB N-terminal acetylase in Saccharomyces cerevisiae. J. Microbiol. 52, 842–848 (2014).

21. Oishi, K., Yamayoshi, S., Kozuka-Hata, H., Oyama, M. & Kawaoka, Y. N-terminal acetylation by NaB is required for the shutoff activity of influenza A virus PA-X. Cell Rep. 24, 851–860 (2018).

22. Fernandez-Ayela, A. et al. Mutation of an Arabidopsis NatB N-alpha-terminal acetylation complex component causes pleiotropic developmental defects. PLoS ONE 8, e66287 (2013).

23. Huber, M. et al. NatB-mediated N-terminal acetylation affects growth and abiotic stress responses. Plant Physiol. 182, 792–806 (2019).

24. Caesar, R., Warringer, J. & Blomberg, A. Physiological importance and identification of novel targets for the N-terminal acetyltransferase NatB. Eukaryot. Cell 5, 368–378 (2006).

25. Ametazzarzu, A., Larrea, E., Civeira, M., Prieto, J. & Aldabe, R. Implication of human N-alpha-acetyltransferase 5 in cellular proliferation and carcinogenesis. Oncogene 27, 7296 (2008).

26. Neri, L. et al. NatB-mediated protein N-terminal acetylation is a potential therapeutic target in hepatocellular carcinoma. Oncotarget 8, 40967 (2017).

27. Yasuda, K., Takahashi, M. & Mori, N. Mdm20 modulates actin remodeling through the mTORC2 pathway via its effect on rictor expression. J. Biol. Chem. 286, 37002–37010 (2011).

28. Ohyama, K., Yasuda, K., Onga, K., Kakizuka, A. & Mori, N. Spatio-temporal thermostability. Nat. Struct. Mol. Biol. 11, 1080 (2009).

29. Ametazzarzu, A. et al. Characterization of the human N-alpha-terminal acetyltransferase B enzymatic complex in BMC proceedings 3 S4 (BioMed Central, 2009).

30. Hong, H. et al. Molecular basis of substrate specific acetylation by N-terminal acetyltransferase NatB. Structure 25, 641–649 (2017). c3

31. Knorr, A. G. et al. Ribosome export factor Arx1 bound at the exit tunnel. Cell 146, 1127 (2013).

32. Liszczak, G. et al. Molecular basis for N-terminal acetylation by the NatB complex. J. Biol. Chem. 286, 4464–4476 (2011).

33. Liszczak, G., Arnesen, T. & Marmorstein, R. Structure of a ternary Naa50p acetyltransferase NatB enzymatic complex. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 213–221 (2010).

34. Knorr, A. G. et al. Structure of the human ribosomal tunnel exit complex with the flexible rRNA expansion segments. EMBO Rep. 18, 10088 (2012).

35. Foy, H. V. et al. Design, synthesis, and kinetic characterization of protein N-terminal acetyltransferase inhibitors. ACS Chem. Biol. 8, 1121–1127 (2013).

36. Wild, K. et al. MetAP-like Ebp1 occupies the human ribosomal tunnel exit and recruits the NatA complex. Nat. Commun. 8, 368 (2017).

37. Van Damme, P. et al. Proteome-derived peptide libraries allow detailed analysis of the substrate specificities of Naa-acetyltransferases and point to hNaa10p as the post-translational acetylation site of the human ribosomal tunnel exit complex with the flexible rRNA expansion segments. J. Biol. Chem. 286, 4464–4476 (2011).

38. Ametazzarzu, A. et al. Insight into structure and assembly of the nuclear pore complex by utilizing the genome of a eukaryotic thermometer. Cell 146, 277–289 (2011).

39. Knorr, A. G. et al. Ribosome-NatA architecture reveals that rRNA expansion through the mTORC2 pathway via its effect on rictor expression. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 213–221 (2010).

40. Drazic, A. et al. The Arabidopsis Na-acetyltransferase NAA60 locates to the hNaa10p as the post-translational acetylation site of the human ribosomal tunnel exit complex with the flexible rRNA expansion segments. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 213–221 (2010).

41. Foy, H. V. et al. Design, synthesis, and kinetic characterization of protein N-terminal acetyltransferase inhibitors. ACS Chem. Biol. 8, 1121–1127 (2013).

42. Liszczak, G., Arnesen, T. & Marmorstein, R. Structure of a ternary Naa50p acetyltransferase NatB enzymatic complex. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 213–221 (2010).

43. Liszczak, G., Arnesen, T. & Marmorstein, R. Structure of a ternary Naa50p acetyltransferase NatB enzymatic complex. Acta Crystallogr. Sect. D: Biol. Crystallogr. 66, 213–221 (2010).

44. Drazic, A. et al. NAA80 is actin remodeling through the mTORC2 pathway via its effect on rictor expression. J. Biol. Chem. 286, 37002–37010 (2011).

45. Schrödinger, L. C. The PyMOL molecular graphics system. Version 1, 8 (2015).

46. Becker, M. M., Lapouge, K., Segnit, B., Wild, K. & Sinning, I. Structures of human SRP72 complexes provide insights into SRP RNA remodeling and ribosome interaction. Nucleic Acids Res. 45, 470–481 (2017).

47. Andrew Skaff, D. & Mizzorno, H. M. A visible wavelength spectrophotometric assay suitable for high-throughput screening of 3-hydroxy-3-methylglutaryl-CoA synthase. Anal. Biochem. 396, 96–102 (2010).

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Richardson, T. M. & Polphobity: all-atom structure validation for macromolecular cryocrystallography. Acta Crystallogr. D. 66, 12–21 (2010).

Krisinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797 (2007).

Andrew Skaff, D. & Mizzorno, H. M. A visible wavelength spectrophotometric assay suitable for high-throughput screening of 3-hydroxy-3-methylglutaryl-CoA synthase. Anal. Biochem. 396, 96–102 (2010).

Author contributions D.L. designed, performed, and analyzed experiments, collected and processed X-ray diffraction data, analyzed all data, synthesized the bisubstrate analog, wrote the manuscript. J.K. performed crystallization screening, collected and processed X-ray data collections were performed at beamlines X06DA PXIII (SLS, Paul Scherrer Institut, Villigen, Switzerland) and beamline P41 (EMBL Hamburg at the PETRA III storage ring, DESY, Hamburg, Germany), respectively. We acknowledge the data storage service SDS@hhd supported by the Ministry of Science, Research and the Arts Baden-Württemberg (MWK) and the German Research Foundation (DFG) through grant INST 35/1314-1 and INST 35/1503-1 FUGG. I.S. is an investigator of the Cluster of Excellence: CellNetworks. This work was funded by the German Research Foundation (DFG) through the Leibniz program (SI 5866/1-1) and Project-ID 201348542 – SFB 1036 (TP22) to I.S., and by an ERC Starting Grant (336367) to M.K.

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