N-terminal acetylome analyses and functional insights of the N-terminal acetyltransferase NatB

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Protein N-terminal acetylation (Nt-acetylation) is an important mediator of protein function, stability, sorting, and localization. Although the responsible enzymes are thought to be fairly well characterized, the lack of identified in vivo substrates, the occurrence of Nt-acetylation substrates displaying yet uncharacterized N-terminal acetyltransferase (NAT) specificities, and emerging evidence of posttranslational Nt-acetylation, necessitate the use of genetic models and quantitative proteomics. NatB, which targets Met-Glu-, Met-Asp-, and Met-Asn-starting protein N termini, is presumed to Nt-acetylate 15% of all yeast and 18% of all human proteins. We here report on the evolutionary traits of NatB from yeast to human and demonstrate that ectopically expressed hNatB in a yNatB-Δ yeast strain partially complements the *natB*-Δ phenotypes and partially restores the yNatB Nt-acetylome. Overall, combining quantitative N-terminomics with yeast studies and knockdown of hNatB in human cell lines, led to the unambiguous identification of 180 human and 110 yeast NatB substrates. Interestingly, these substrates included Met-Gln- N-termini, which are thus now classified as in vivo NatB substrates. We also demonstrate the requirement of hNatB activity for maintaining the structure and function of actomyosin fibers and for proper cellular migration. In addition, expression of tropomyosin-1 restored the altered focal adhesions and cellular migration defects observed in hNatB-depleted HeLa cells, indicative for the conserved link between NatB, tropomyosin, and actin cable function from yeast to human.

N-terminal acetylation (Nt-acetylation) represents one of the most common protein modifications in eukaryotes, occurring on ~50–70% of yeast proteins and on 80–90% of human proteins (1–3), but rarely on prokaryotic proteins. In eukaryotes, nascent protein chains typically start with an initiator methionine, which is cleaved of if the second residue has a radius of gyration of 1.29 Å or less (4). Protein Nt-acetylation is believed to occur cotranslationally upon protrusion of the growing polypeptide chain from the ribosomal polypeptide exit tunnel (5). The large number and diversity of Nt-acetylation substrates stems largely from the existence of enzymatically different NatA-NatF N-terminal acetyltransferases (NATs) (2, 3, 6, 7). Each NAT of this family exhibits strong preferences for specific N-terminal residues, and (at least) one or two next amino acids required to steer Nt-acetylation by a NAT (2, 6). An exception is NatD, which requires 30–50 specific amino acids for in vivo Nt-acetylation of its substrates (8).

Until recently, the biological consequences of protein Nt-acetylation have remained enigmatic. However, an increasing number of reports indicate that Nt-acetylation is essential for higher eukaryotes, but less critical for normal growth in yeast (9–12). Functionally, Nt-acetylation was found to regulate a variety of protein features (13), including the degradation (at least in yeast) of some Nt-acetylated proteins by a new branch of the N-end-rule pathway (14) and the ability of Nt-acetylation to inhibit protein translocation into the endoplasmic reticulum (15). The major NATs, NatA, -B, and -C are heterodimers or heterotrimers. In particular, NatB comprises the catalytic subunit Naa20p (Nat3p) and the auxiliary subunit Naa25p (Mdm20p) (6). Although NatA acetylates Ser-, Thr-, Ala-, Gly-, and Val- N termini follow initiator Met (iMet) processing by methionine aminopeptidases or MetAPs (2, 4, 16), NatB acetylates Met residues followed by an acidic or Asn residue (2, 9, 10, 12) or, in the case of NatC, Met followed by a hydrophobic residue (7).

The yeast *naa20*-Δ and *naa25*-Δ deletion mutant strains display the most prominent phenotypes of all studied NAT subunit deletions, including temperature sensitivity (ts^-) and increased sensitivity to salt and other osmotic agents, caffeine, anti-microtubule, and DNA damage drugs, as well as to several other chemicals (7). These strains further showed reduced mating, abnormal morphology, cell polarity and cytoskeleton function, defects in mitochondrial division, and vacuolar segregation. Some of these defects are primarily because of lack of Nt-acetylation in two essential cytoskeleton proteins, actin and tropomyosin (10), and probably of certain, yet unidentified DNA repair factors and cell cycle proteins (17, 18). Of further note is that differences between the *ynaa20*-Δ and *ynaa25*-Δ phenotypes were reported, which points to nonoverlapping functions of the NatB subunits (18). Besides minor alterations in overall protein expression (e.g., increased expression of proteins implicated in ribosome biogenesis, cytokinesis and budding) and the direct effects mediated by perturbation of protein Nt-acetylation, *ynaa20*-Δ deletion causes elevated levels of protein phosphorylation (19). Interestingly, although very few proteins display a conserved NatB-type substrate specificity (19), proteins with cell cycle functions are overrepresented in the pool of predicted yeast NatB substrates (18). In agreement with this representation pattern, the silencing of *hNAA20* or *hNAA25* in human cell lines induces growth arrest, indicating a role of hNatB in cell cycle progression (12), among others, by altered induction levels of (anti-)proliferative genes (9, 12, 20).

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Nt-acetylation of tropomyosin-1 stabilizes the coiled-coil α-helix at its N terminus and is required for high affinity binding of tropomyosin to actin (21, 22). In this way, tropomyosin forms continuous head-to-tail noncovalent filaments that interact with the actin filaments, thereby modulating actin interactions with other actin-binding proteins (21). For yNatB, several substrates have been reported, including tropomyosin-1 (Tpm1), two ribosomal proteins (23), the 20S proteosomal subunit (24), the stress-induced carboxypeptidase inhibitor Tfs1p (17), and the 59 yNaa20p substrates recently identified by mass spectrometry (19). However, thus far no substrates of the hNatB complex (12) have been identified, and its substrate specificity has only been inferred from in vitro Nt-acetylation assays using selected oligopeptide substrates (9, 12).

We have here studied the NatB specificity and identified its substrates by quantitative analyses of different yeast and human Nt-acetylomes using N-terminal combined fractional diagonal chromatography (COFRADIC) (1, 25). hNatB was shown to be active in yeast; however, the reduction in the degree of Nt-acetylation of the hNatB yeast compared with the control yeast Nt-acetylome agrees with the partial rescue of yNatB-deletion phenotypes. This finding, together with hNatB knockdown studies in human cells, univocally reveals that the yeast and human NatB substrate specificity is largely conserved; however, our quantitative Nt-acetylation studies demonstrate that hNatB has evolved to more effectively Nt-acetylate Met-Gln- N termini. The remaining levels of Nt-acetylation for certain NatB-type substrate N termini in the yNatB deletion strain further point to redundancy among the NATs. In addition, we observed that NatB activity is maintained from yeast to human as a key component for supporting actin cytoskeleton structure and function, because down-regulation of hNatB subunit expression blocks actomyosin and focal adhesion formation and consequently inhibits cellular migration.

Results

Human *hNAA20-hNAA25* Expression in Yeast Partially Rescues the Yeast *ynaa20-Δ ynaa25-Δ* Phenotypes. To determine if the yeast and human NatB complexes have similar substrate specificities and if ectopically expressed hNatB is capable of suppressing the yeast *natB*-Δ phenotypes, we cloned *hNAA20* and *hNAA25* into yeast expression vectors. We found that hNatB overexpression only partially suppressed the *natB*-Δ phenotypes (Fig. 1). Whereas sensitivity of the *natB*-Δ yeast to calcium chloride, camptothecin, and hydroxyurea was completely or nearly completely suppressed, sensitivity to salt, caffeine, and ethylmethanesulfonate was not, in addition to partial complementation of sensitivities to diethylene glycol and thiabendazole. Furthermore, heterologous combination of *hNAA20* and y*MDM20* (*yNAA25*) did not produce active NatB and did not complement y*natB*Δ phenotypes.

Human *hNAA20-hNAA25* and Yeast *yNAA20-yNAA25* Encode NATs That Display Overlapping and Divergent Functions. To elucidate the in vivo Nt-acetylomes of yNatB and hNatB and to delineate potential differences in their in vivo substrate specificity profiles in yeast, quantitative N-terminal COFRADIC analyses were performed (1, 25), and the Nt-acetylation states of wild-type, yNatB-Δ and y[hNatB] yeast proteomes were compared. To distinguish between in vivo Nt-acetylated and non–Nt-acetylated N termini, we used chemical in vitro $^{13}C_2D_3$-acetylation, which introduces a 5-Da mass spacing between the non–Nt-acetylated and Nt-acetylated N-terminal peptide form and thereby allows the calculation of the extent of Nt-acetylation (1, 26). Overall, in the three yeast strains analyzed, 1,623 unique yeast N termini originating from 1,321 yeast proteins were identified (Table S1). Here, an N terminus is defined as either a peptide that is in vivo Nt-acetylated or in vitro $^{13}C_2D_3$-acetylated (i.e., an in vivo non–Nt-acetylated N terminus) and starts at position 1 or 2 of the protein sequence as stored in the SwissProt database, or an in vivo Nt-acetylated peptide with a starting position beyond 2.

Of these 1,623 N termini, 1,102 started at position 1 or 2, and 521 started beyond position 2 (Table S1). The latter indicate

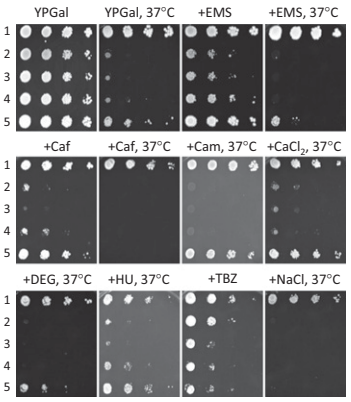

Fig. 1. Yeast yNatB-Δ strain phenotype suppression by overexpression of hNatB. Serial 1/10 dilutions of the following isogenic series of strains were grown at 30 °C and 37 °C on different media for 3 d: yNatB, B-13480 (row 1); yNatB-Δ, B-16209 (row 2); yNatB-Δ+vector, B-16292 (row 3); y[*hNAA20*], B-16293 (row 4); and y[hNatB], B-16266 (row 5). Standard medium, YPGal [1% (wt/vol) bacto-yeast extract, 2% (wt/vol) Bacto-peptone, and 2% (wt/vol) galactose] containing the following amounts of different agents was used: 0.9 M sodium chloride (NaCl); 0.3 M calcium chloride ($CaCl_2$); 6.7% (wt/vol) diethylene glycol (DEG); 0.15% caffeine (Caf); 50 μg/mL thiabendazole (TBZ); 0.075% ethyl methanesulfonate (EMS). Other phenotypes were determined with synthetic complete medium (SD) containing the following: 75 mM hydroxyurea (HU) and 4 μg/mL camptothecin (Cam).

alternative translation events or posttranslational Nt-acetylation (19, 27–29). Of the protein N termini, 414 (25%) were identified in all three N terminomes. For all N termini identified, the difference in the degree of Nt-acetylation among the different yeast strains was calculated. As anticipated, considerable variations in the degree of Nt-acetylation (here defined as a minimum difference of 10% in the degree of acetylation, as defined in ref. 3) of yeast N termini were almost exclusively confined to the NatB-type of N termini. Of note is that in addition to the canonical NatB substrate specificity (i.e., Met-Asp-, Met-Glu-, and Met-Asn- N termini), Met-Gln- substrate specificity was observed for yNatB as well as for hNatB. In total, 261 NatB-type substrate N termini (or 16% of all N termini) were identified [i.e., 261 of the 1,175 or 22% of all Met-starting SwissProt (version 57.8) annotated yeast NatB type N termini were identified (3)], and for 110 of these (or 42%), human and/or yeast NatB-dependency for Nt-acetylation was found (Table S1). Here, NatB substrate N termini were considered when the corresponding peptides were either found to be completely nonacetylated in the yNatB-Δ strain compared with the y[hNatB] or yNatB strain, or displayed a shift in Nt-acetylation larger than 10% compared with the control sample (Fig. 2*A* and Table S2). In this way, 106 N termini were further classified as genuine yeast or hNatB substrate N termini (Table S2). The fact that nearly all assignable NatB-type N termini were determined to be Nt-acetylated by NatB further indicates that the vast majority of the 151 unassigned NatB-type N termini also represent NatB substrate N termini (Table S1).

From all of the NatB-type substrates identified in the control setup, only nine N termini were incompletely Nt-acetylated and three non–Nt-acetylated, and these were mainly Met-Gln- N termini. By and large, of all of the NatB substrate N termini found to be acetylated by the hNatB-complex, the degree of Nt-acetylation was found to be reduced compared with the control yNatB setup (i.e., Nt-acetylation was only partial). This finding indicates that NatB-specific substrate Nt-acetylation was only partially restored by hNatB, which agrees with the fact that certain yNatB-Δ–specific phenotypes were not complemented in the y[hNatB] strain (Fig. 1). However, several Met-Gln-starting N termini are an exception to this, because their hNatB-mediated acetylation levels were increased compared with the control setup (Table S2). This finding hints to the fact that hNatB has

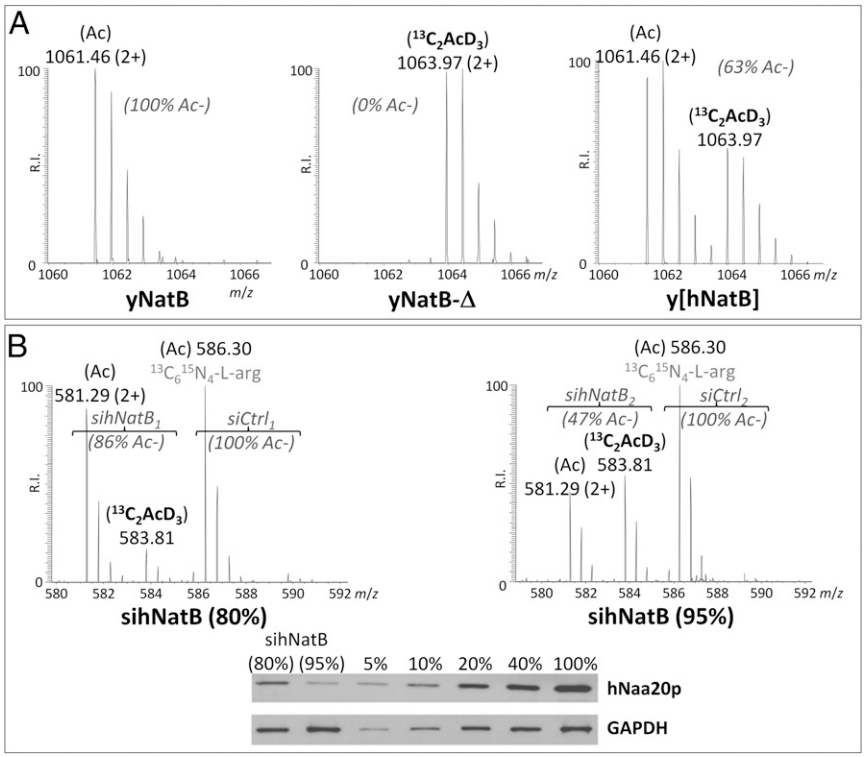

Fig. 2. Expression of hNatB in y[hNatB] only partially restores the N-terminal acetylation status of yNatB substrates and knockdown of hNatB affects N-terminal acetylation in HeLa cells. (*A*) MS-spectra from the MN- starting N-terminal peptide (doubly charged precursor) of the Heat shock factor protein YPL199C (^{1}MNNAANTGTTNESNVSDAPR^{20}) demonstrate this to be a genuine yNatB substrate N termini as it was found to fully (*Left*) and 0% Nt-acetylation (*Center*) in control and yNatB-Δ yeast proteomes, respectively. Two distinguishable isotopic envelopes could be distinguished in the y[hNatB] yeast strain [i.e., the acetylated (Ac) and $^{13}C_2$ and trideutero-acetylated forms ($^{13}C_2AcD_3$), *Right*] indicative for the fact that this N terminus is only partially (63% Nt-acetylation) in vivo Nt-acetylated (*Right*). (*B*) HeLa cells cultivated in $^{13}C_6{}^{15}N_4$ L-arginine were transfected with 10 nM si-nontargeting control and cells cultivated in $^{12}C_6$ L-arginine were transfected with 10 nM si*hNAA20*/si*hNAA25* pool. MS spectra of the N terminus of the Fragile X mental retardation-1 protein (^{1}MEELVVEVR^9) are shown. The peptide was fully acetylated in both control setups but was only partially acetylated in both knockdown samples analyzed. Upon pronounced hNatB knockdown, the level of Nt-acetylation drops from 86–47%, but the total concentration of the N terminus remained unaffected in the two samples analyzed. (*Lower*) An estimation of the hNatB knockdown efficiency. Cellular extracts from both COFRADIC experiments and several dilutions of HeLa cellular extracts (100–5%) were subjected to SDS/PAGE and immunoblotted with anti-hNaa20 and anti-GAPDH antibodies.

evolved to more efficiently acetylate Met-Gln-starting N termini, in line with the fact that in humans such N termini are significantly more Nt-acetylated than in yeast (1). Of further note is that in the NatB deletion strain, 12 N termini remained partially Nt-acetylated. Of these strains, five N termini started with Met-Gln-, suggesting that a redundant yNAT is active on (a subset) of these N termini (Table S2), which again indicates that (certain) NATs display overlapping substrates/substrate-specificities (3).

Protein Nt-Acetylation in HeLa Cells After RNAi-Mediated hNatB Perturbation. To probe for the natural substrate repertoire of hNatB, two independent and differential N-terminal COFRADIC experiments were performed comparing the Nt-acetylomes of control HeLa cells with those of siNatB-transfected cells (sihNatB) with hNatB knockdown efficiencies of respectively 80% (setup 1) and 95% (setup 2) (Fig. 2*B*). Here, in vitro $^{13}C_2D_3$-acetylation was combined with differential L-Arg stable isotope-labeling by amino acids in cell culture, which allows for the calculation of the extent of Nt-acetylation and for the relative quantification of N-terminal peptides. The siNatB and siCtrl samples were labeled with a light ($^{12}C_6$-L-Arg) and heavy isotopic variant of L-Arg ($^{13}C_6{}^{15}N_4$-L-Arg), respectively. Following LC-MS/MS analysis, we identified 2,102 unique N termini (Table S3). Of these, 595 N termini (28.3%) displayed a NatB-type of substrate specificity [Met-Asp-, Met-Glu-, Met-Asn-, and Met-Gln- N termini (Table S3)], and 163 (27.4% or 91% of all siNatB affected N termini) displayed a significant reduction in Nt-acetylation upon NatB knockdown and were thus assigned as in vivo hNatB substrate N termini (Fig. 2*B* and Table S4). Remarkably, few unknown Nat-type substrate N termini were also affected in their Nt-acetylation status (including Met-Gly-, Met-Ser-, Met-Val-, and Met-Thr- N termini) (Table S4). Furthermore, substrate N termini identified in both sihNatB HeLa proteomes were more affected upon more efficient knockdown of NatB (i.e., 9% vs. 38.8% of the potential NatB-type substrates displayed a reduced Nt-acetylation), strengthening the fact that suboptimal NatB-type N termini are primarily affected upon NatB perturbation (Table S4).

Analogous to the subtle changes in protein expression observed in the *ynaa20p-Δ ynaa25-Δ* strain (19), based on the MS-signal intensities of the identified N-terminal peptides, few differences in protein expression were found upon NatB knockdown (Table S5). Of note here is the fact that the immature, Met-retaining variant of β-actin (MDDD-form) was significantly affected in its Nt-acetylation level (i.e., from 100–75% Nt-acetylated) and was thus identified as a hNatB-substrate (Table S4). Based on the signal intensities of its corresponding N terminus, expression of the mature form of β-actin (DDD-form) was not found to be significantly regulated upon NatB knockdown. Conversely, the immature MEEE-variant of γ-actin, of which the Nt-acetylation level was unaffected by NatB knockdown, was found to be significantly up-regulated ($P \leq 0.01$) in both sihNatB setups—4- to 10-fold when going from 80–95% of knockdown—but expression of its mature EEE-variant remained unaffected. hNaa10p and hNatA were recently found to Nt-acetylate the mature actin N termini (30) and, to complete the current view on actin Nt-

acetylation, we pursued if hNatB can Nt-acetylate the immature β- and γ-actin N termini. Quantitative HPLC-based in vitro acetylation assays indeed demonstrated Nt-acetylation of the immature β-actin N-terminal peptide (MDDD-) by immunoprecipitated hNatB but not of the mature β- and γ-actin N termini (Fig. S1). These results indicate that the observed defects in actin cytoskeleton organization (see below) are not caused by direct Nt-acetylation of the most common γ/β-actin forms (the acidic N-termini EEE-/DDD-).

hNatB Down-Regulation Affects Actin Cytoskeleton Structure and Function. Destabilization of yeast actin cables caused by the absence of Nt-acetylation of tropomyosin and actin was observed in the yeast *naa20*-Δ strain (10, 31). To determine whether hNatB activity is required in human cells for the maintenance of the integrity of actin microfibrils, we inhibited the expression of either *hNAA20* or *hNAA25* in HeLa cells and examined the F-actin network by labeling it with fluorescent phalloidin. In each case, we observed a clear reduction of actin stress fibers in HeLa cells (Fig. 3*A*). To determine if other proteins associated with actin microfibrils are relocalized as a consequence of hNatB down-regulation, we visualized the distributions of myosin IIA, caldesmon and α-actinin (Fig. 3*B* and Fig. S2) (32). hNaa20p or hNaa25p depletion delocalized myosin II and reorganized the localization of α-actinin and caldesmon, indicating that, as previously described for its yeast ortholog yNatB, hNatB activity is essential for maintaining stable actomyosin fibers.

Stress fibers terminate at extracellular attachment sites, such as focal adhesions, where they are linked via integrins to the extracellular matrix. Focal adhesions are multiprotein complexes that grow and change in composition in response to mechanical tension supplied either through actomyosin fibers or by external forces applied to the cell (33). Because hNatB knockdown disorganizes actomyosin fibers, we wanted to determine if hNatB knockdown also affects the formation of focal adhesions. We analyzed the cellular localization of vinculin and paxilin (Fig. 3*B* and Fig. S2), two intracellular focal adhesion proteins, and quantified the number and size of focal adhesions in HeLa cells after depletion of either of the two hNatB subunits. In both cases we observed a decrease in the number and size of focal adhesions per cell suggesting that hNatB activity is necessary for focal adhesion formation (Fig. 3 *C* and *D*).

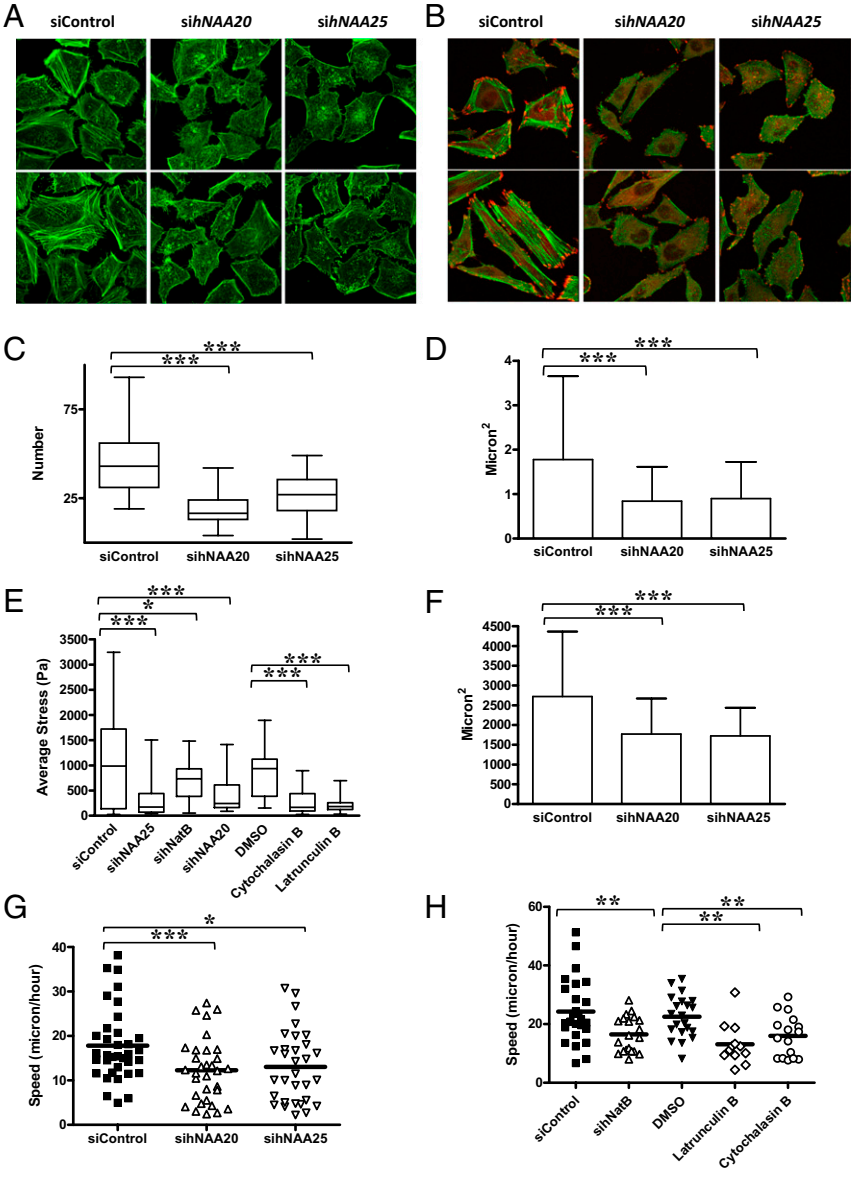

Fig. 3. hNatB depletion perturbs actin cytoskeleton and focal adhesion organization reducing cellular motility. (*A* and *B*) Immunocytochemical analysis of HeLa cells transfected for 96 h with nontargeting control siRNA (siControl), hNaa20- and hNaa25-targeting siRNAs (si*hNAA20* and si*hNAA25*). Immunostaining is shown for actin stress fibers visualized with GFP-phalloidin (*A*), myosin IIA localization (green) (*B*), and vinculin (red) as focal adhesion marker (*B*). The focal adhesions observed after each treatment where characterized in more detail quantifying the number (siControl $n = 852$, si*hNAA20* $n = 364$, si*hNAA*25 $n = 344$) (Magnification: 630×) (*C*) and focal adhesion mean area (siControl $n = 19$, si*hNAA20* $n = 20$, si*hNAA25* $n = 13$) (*D*) detected per cell. Two-dimensional traction-force microscopy measurements were performed on HeLa cells treated for 5 d with nontargeting control siRNAs (siControl $n = 30$) or siRNAs targeting hNatB subunits (hNaa20 $n = 32$, hNaa25 $n = 32$ or hNaa20+hNaa25 $n = 32$). Cells were treated also for 5 d with DMSO ($n = 36$), latrunculin B 0.2 μM ($n = 28$), or cytochalasin B 0.2 μM ($n = 29$) before analyzing traction force average stress of the whole cell (*E*). (*F*) Quantification of surface occupied by the cells after depletion of either of the two hNatB subunits (si*hNAA20* $n = 71$, si*hNAA25* $n = 66$) and control HeLa cells (siControl $n = 63$). (*G*) Graph of individual cell speeds calculated for cells transfected with control siRNAs (siControl $n = 35$), *hNAA20* siRNAs (si*hNAA20* $n = 32$), or *hNAA25* siRNAs (si*hNAA25* $n = 31$). (*H*) Regulation of cell speed in the presence of latrunculin B 0.2 μM ($n = 12$), cytochalasin B 0.2 μM ($n = 12$), solvent (DMSO $n = 10$), or siRNA transfected cells (siControl $n = 13$ and sihNatB (si*hNAA20* + si*hNAA25*) $n = 14$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, versus paired control (unpaired Student *t* test). Bar graphs show mean ± SD. Results shown are representative of two separate experiments.

Two-dimensional cell motility depends on forces generated from the dynamic remodeling of the actomyosin cytoskeleton, and these forces are transmitted through focal adhesions to the extracellular matrix. We postulated that the contribution of hNatB to the maintenance of actomyosin fibers and the formation of focal adhesions would be critical for the cell's ability to generate and transmit force to the extracellular matrix. By comparing whole-cell traction forces in HeLa cells with or without reduced expression of the hNatB subunits, we found that the forces transmitted to the extracellular matrix were significantly decreased in cells depleted of hNatB (Fig. 3*E*), resembling the effect observed when cells are treated with latrunculin B and cytochalasin B, compounds that impair actin polymerization (Fig. 3*E*). The cellular traction forces exerted by the cytoskeletal machinery on its extracellular matrix are directly correlated with cell area (34) and, in part, control cell physiology. A significant reduction in the cellular spreading area was detected after knocking down either of the two hNatB subunits compared with control cells (Fig. 3*F*), correlating the effects observed on focal adhesion formation and traction force generation.

As indicated above, cell motion requires the transmission of the traction forces developed in actomyosin microfilaments to the substrate via focal adhesion complexes that connect actin microfilaments to the extracellular matrix (35). Experimental disruption of the generation of such intracellular forces impedes a range of functions, including directed cell migration. Time-lapse imaging was therefore used to determine if the reduction of the cellular traction forces that occurs after hNatB down-regulation affects cellular migration. Independent of targeting either or both hNatB complex subunits, a significant decrease in the speed of cellular migration was observed (Fig. 3 *G* and *H*). Consistent with previous reports, treatment of HeLa cells with latrunculin B or cytochalasin B interfered with cell migration (Fig. 3*H*), similar to the effect observed when hNatB levels are reduced. In addition, we analyzed the expression of myosin light-chain kinase (MLCK), a protein kinase that regulates stress fiber formation, focal-adhesion constitution, and cell migration (36). After treating HeLa cells with latrunculin B and cytochalasin B, MLCK was down-regulated, in analogy with the effect observed when hNatB was down-regulated (Fig. S3).

Tpm1 Overexpression Partially Restores Actin Cytoskeleton Dysfunction Caused by hNatB Knockdown. It was previously shown that NatB-mediated Nt-acetylation of Tpm1 is important for the formation of stable yeast F-actin structures (10, 31). In the case of Tpm1, Nt-acetylation can be mimicked by the expression of an amino-terminally extended form of Tpm1 in vitro (37). Therefore, and because our proteomics data indicated only a partial reduction in the degree of Nt-acetylation of hNatB substrates when knocking down hNatB, we reasoned that overexpression of Tpm1 in hNatB-depleted HeLa cells may restore the function of actomyosin microfibrils. Therefore, wild-type human Tpm1 or human Tpm1 with an amino-terminal tail (MAS- N terminus), functionally mimicking Nt-acetylation (37), was overexpressed in HeLa cells with reduced hNatB activity. Quantification of the number and size of focal adhesions per cell showed that expression of either Tpm1 or MAS-Tpm1 in control cells reduced the size and number of focal adhesions, albeit to a lesser extent than caused by reduced hNatB expression (Fig. S4 *A* and *B*). In contrast, overexpression of human Tpm1, wild-type or mutant, in HeLa cells with reduced hNatB expression levels significantly restored the number of focal adhesions and the focal adhesion area to levels similar to those observed in cells overexpressing human Tpm1 in control cells (Fig. S4 *A* and *B*).

To determine if Tpm1 overexpression can restore cellular motility after hNatB inhibition, we analyzed the speed of cellular migration. In agreement with previous studies demonstrating that tropomyosin overexpression reduces cellular motility (38–40), we observed that human Tpm1 expression significantly diminished the speed of HeLa cell migration (Fig. S4 *C* and *D*). However, when Tpm1 was expressed in hNatB-depleted HeLa cells, there was a reconstitution of cellular migration, in accordance with the recovery of focal adhesion formation observed in these cells. Interestingly, Tpm1 expression could not restore cellular movement in latrunculin B-treated cells (Fig. S4*D*). Consequently, the Tpm1 defect is the main determinant of the effects of hNatB depletion on the structure and function of the actin cytoskeleton.

Discussion

hNatA complementation studies in yeast revealed that the Nt-acetylomes and levels of Nt-acetylation of hNatA substrates were nearly indistinguishable from those of yNatA N termini (1). In contrast, our phenotype and proteomics data presented here demonstrated only a partial rescue of yNatB function by heterologously expressed hNatB, and may hint to the fact that, given the observed general decrease in the overall levels of Nt-acetylation of NatB substrates, the Nt-acetylation levels of a (few) specific NatB substrates get critically low for their proper activity, explaining the observed phenotypic differences between the control and y[hNatB] strains and in-line with previous comparative phenotypic substrate analysis (18). The not fully effective functioning of hNatB in yeast might be caused by an altered assemblage or association with yeast ribosomes (41), (subtle) differences in substrate specificities, or the additional requirement of cofactors and posttranslational protein modifications. In fact, the observed partial Nt-acetylation of NatB substrates by hNatB in yeast is in-line with the Nt-acetylation effects observed upon knocking down NatB in HeLa cells. Striking, however, is that an additional reduction of hNatB levels in HeLa cells by only 15% (i.e., from 80–95%) increased the substrate identification rate fourfold, with to up to 39% of the potential substrate repertoire identified as genuine hNatB substrates. Closer examination of the hNatB substrate subcategories reveals that only about 27% of the Met-Asp- (39/146) and 31% of the Met-Glu-type N termini (75/244) are affected, but the effect of hNatB knockdown on Nt-acetylation of Met-Gln- (13/29 or 45%) and Met-Asn- (39/53 or 74%) N termini is more pronounced. Hence, these data indicate that hNatB appears to prefer Met-Asp- and Met-Glu-starting polypeptides, followed by Met-Gln- and Met-Asn-starting ones. The more efficient Nt-acetylation of Met-Gln starting N termini by hNatB compared with yNatB is reflected by the increase in Nt-acetylation of Met-Gln- N termini in higher eukaryotes, a substrate specificity profile shared with the higher eukaryotic specific NAT, NatF (3), and further demonstrates the existence of substrate redundancy among NATs.

The processes that regulate the actin cytoskeleton structure and function are complex. We have here analyzed the relationship between hNatB-mediated protein Nt-acetylation and the actin network in the course of cellular migration. Our results indicate that a defect in hNatB-mediated Nt-acetylation disrupts actin stress fibers and focal adhesions, thereby impairing cellular movement. Specifically, hNatB depletion decreased Nt-acetylation of unprocessed β-actin. Interestingly, NatB mediated Nt-acetylation of Nt-unspliced tropomyosin and iMet-retaining forms of actin is conserved in yeast and mammals. NatB-deficient yeast strain exhibit defects in actin cable formation similar to observed in the strains with actin and tropomyosin mutations (10, 31). These findings suggest that such defects are caused by the lack of acetylated actin and tropomyosins.

Consistent with experimental conditions that cause loss of stress fibers, depletion of one of both hNatB subunits reduced the amount and size of focal adhesions (42) and a significant decrease in cellular traction forces, also observed when actin polymerization or myosin contractility is altered. Such traction forces are mediated by molecules that regulate stress fiber and focal adhesion assembly such as nonmuscle myosin II and MLCK (43). Both mechanisms were affected by hNatB down-regulation, as indicated by disruption of stress fibers, focal adhesions, and MLCK activity (Fig. 3 and Fig. S3).

Previous studies have observed that an increment in tropomyosin expression partially restores functional defects in actin

stress fibers (31, 37). In contrast, Tpm1 overexpression in wild-type HeLa cells significantly reduced the area and number of focal adhesions, in agreement with other expressed tropomyosin isoforms (38) and suggestive of a compromised Tpm1 function, most probably by its reduced Nt-acetylation. Increasing the amount of Tpm1 attenuated the effects of hNatB inhibition and restored the proper formation of focal adhesions. The effect of Tpm1 overexpression was associated with the recovery of cellular motility when hNatB is inhibited. This recovery was not observed when Tpm1 was overexpressed in cells that were treated with latrunculin B, which caused the disorganization of the actin network and impaired cellular motility. The expression of Tpm1 with a dipeptide N-terminal extension, mimicking the structural effects of Nt-acetylation (37), restored the cytoskeletal and motility defects induced by hNatB depletion (Fig. S4).

Tropomyosin expression increased or decreased focal adhesion area (38–40), consistent with the idea that maximal migration rates require the specific organization of the focal adhesions and actin filaments. Similarly, our findings provide evidence indicating that the tight regulation of tropomyosin and the amount of acetylated Tpm1 is necessary for maximal cellular motility (Fig. S4).

Based on previous data and our present data, we suggest that Tpm1 Nt-acetylation is necessary for its interaction with other proteins to maintain the actin cytoskeletal structure and function in mammalian cells, and plays an important role to regulate adhesion structures and cell migration. However, we cannot rule out that the lack of hNatB-mediated acetylation of other identified substrates, such as myosin-X and actin filament-associated protein 1 or others, may cause similar phenotypes by presenting altered activities or interactions with other proteins.

Materials and Methods

Information on materials and methods are provided in *SI Materials and Methods*. All original MS data are available on-line at PRoteomics IDEntifications database (PRIDE) (40) (http://www.ebi.ac.uk/pride/) with accession numbers 16372 and 16373.

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