**Naa12 rescues embryonic lethality in Naa10-Deficient Mice in the amino-terminal acetylation pathway**


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Abstract

There is an enormous amount of variation in proteins introduced by co- and post-translational modifications, including N-terminal acetylation (NTA), catalyzed by a set of N-terminal acetyltransferases (NATs). The NatA complex (including X-linked Naa10 and Naa15) is the major acetyltransferase, with 40–50% of all mammalian proteins being potential substrates. However, the overall role of NTA on a whole-organism level is poorly understood, particularly in mammals. Male mice lacking Naa10 show no globally apparent in vivo NTA impairment and, surprisingly, do not exhibit embryonic lethality. Rather Naa10 nulls display increased neonatal lethality, and the majority of surviving undersized mutants exhibit a combination of hydrocephaly, cardiac defects, homeotic anterior transformation (including an extra thoracic rib), piebaldism and urogenital anomalies. The lack of complete embryonic lethality in Naa10-null mice is explained by the discovery of Naa12, a previously unannotated Naa10-like paralogue with NAT activity that genetically compensates for Naa10. Mice deficient for Naa12 have no apparent phenotype, except for decreased fertility, whereas mice doubly deficient for Naa10 and Naa12 display embryonic lethality, thus presenting the complete machinery for NatA-mediated NTA in mouse development.

Introduction

N-terminal (Nt-) acetylation (NTA) is one of the most common protein modifications, occurring co- and post-translationally. Approximately 80% of cytosolic proteins are N-terminally acetylated in humans and ~50% in yeast (1), while NTA is less common in prokaryotes and archaea (2). NTA is catalyzed by a set of enzymes, the N-terminal acetyltransferases (NATs), which transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the free α-amino group of a protein’s N-terminus. To date, eight distinct NATs (NatA – NatH) have been identified in eukaryotes that are classified based on different subunit compositions and substrate specificities (3-5). NTA has been implicated in steering protein folding, stability or degradation, subcellular targeting, and complex formation (6-10). The vital role of NATs and NTA in development has also emerged (11).

NatA, the major NAT complex, targets ~40% of the human proteome, acetylating Ser-, Ala-, Gly-, Thr-, Val- and Cys N-termini after removal of the initiator methionine (1, 5). Human NatA consists of two main subunits, the catalytic subunit N-α-acetyltransferase 10 (NAA10) (Ard1) and the auxiliary subunit NAA15 (Nat1), and a regulatory subunit HYPK (12-14). NAA15 function has been linked to cell survival, tumor progression, and retinal development (15, 16). In addition, Naa10-catalyzed N-terminal acetylation has been reported to be essential for development in many species (11, 17-22), and although NatA is not essential in S. cerevisiae, depletion of Naa10 or Naa15 has strong effects, including slow growth and decreased survival when exposed to various stresses (23, 24).

NAA10 mutations were found to be associated with several human diseases characterized by severe phenotypes, including global developmental defects (11). Among these, the X-linked Ogden syndrome (OS) (25, 26) shows the most severe pathological features such as infant lethality and has reduced NatA catalytic activity. In a Saccharomyces cerevisiae model for the Naa10 Ser37Pro mutant, the mutation impairs NatA complex formation and leads to a reduction in NatA catalytic activity and functionality (27, 28). Further, OS patient-derived cells have impaired NTA in vivo of some NatA substrates (25).
Over the years, many additional pathogenic NAA10 variants have been identified in NAA10 (29-35) and the collection of presenting symptoms is currently referred to as Ogden syndrome or NAA10-related syndrome (36). Other families have also been identified with NAA15 mutations (29-35).

The autosomal NAA10 homolog, NAA11 (ARD2), has been reported to be present in mice and humans, and is co-expressed with NAA10 in human cell lines (37). Therefore, NAA11 could conceivably compensate when NAA10 is reduced or lacking (11). However, NAA11 was only found in testis and placenta in human and gonadal tissues in mouse, while NAA10 showed widespread expression in various tissues in embryos and adults (38). Thus, any functional redundancy or compensation might be limited to certain tissues only.

To elucidate the functional role of Naa10 during development in mice, we used two different Naa10-deficient mouse lines: one, referred to as Naa10 knockout (KO), which was previously reported specifically related to bone density in postnatal day 3 (P3) mice (39), and another denoted as Naa10^tm1a(EUCOMM)Hmgm (Naa10^tm1a), generated in this study. These Naa10-deficient mice exhibit pleiotropic developmental abnormalities at a range of different ages, overlapping with some of the phenotypes seen in human disease involving NAA10 impairment. Because we did not discover major changes in the overall Nt-acetylome in Naa10 KO mice, we hypothesized that there might be a compensating gene in mice, which led us to the identification of a new paralog of Naa10, which we name Naa12. Naa12 is expressed in several organs (liver, kidney, heart and testis) and, like Naa10, binds to Naa15 to mediate NatA activity. Furthermore, lethality was observed in Naa10; Naa12 double-KO mice, which supports the compensatory role of Naa12 in vivo. Thus, we demonstrate that Naa10 is essential for proper development and Naa12, a newly-identified paralog of Naa10, can play a compensatory role in mice.

Results

Naa10 knockout mice can be born, but display pleiotropic developmental defects

To explore the role of Naa10 in development, most analyses were carried out using our Naa10 KO model mice that had been generated previously (39) using a targeting vector deleting Exon1, including the start codon, and Exon2 to Exon4 containing the GNAT domain including the Acetyl-CoA binding motif, which is crucial for Naa10 function. We also generated another Naa10-deficient mouse which we called Naa10^tm1a, expressing β-galactosidase rather than the Naa10 gene (S1A Fig). Naa10 expression was deficient in Naa10^tm1a mice (S1B and S1C Fig). Especially strong β-gal staining was observed during embryonic stages in the brain, heart and spinal cord (S1D Fig). Male Naa10 KO (Naa10^−/−) embryos displayed mild to severe developmental defects compared to wild-type (WT) (Naa10^+/−) embryos. Some Naa10^−/− mice had lower levels of somites and developmental delay. Additionally, some Naa10^−/− embryos had a normal number of somites but were retarded in growth (Fig 1A). Some of the embryos underwent lysis or remained arrested at an earlier stage than embryonic day 10.5 (E10.5), with no turning, an abnormal trunk, and underdeveloped facial features. These phenotypes also reproduced in Naa10^tm1a/− embryos. Next, we assessed whether Naa10 is essential for viability and counted the Mendelian ratios. Both Naa10^−/− and Naa10^tm1a/− mice were under-represented after birth, while there was no significant reduction in the embryonic stage in both mouse lines (S1 and S2 Table). We monitored the pups daily at postnatal day 0 (P0) to postnatal day 3 (P3) and beyond, and the
Congenital heart defects are one of the main causes of infant lethality, and cardiac diseases are a common developmental anomaly in OS patients (31), with some OS males dying in infancy with cardiac arrhythmias (26). Therefore, we investigated whether Naa10 KO affects cardiac development. Development of a four-chambered septated heart is normally complete at E14.5, therefore we examined the cardiovascular system at E14.5. We identified ventricular septal defects (VSD) in several Naa10+/− embryos, as well as concomitant double outflow right ventricle (DORV) at E14.5 (Fig 1D, upper). Ventricular septal defects (VSD) and atrial septal defects (ASD) were also observed at E18.5 (Fig 1D, bottom), and persistent truncus arteriosus (PTA) or DORV, along with concomitant membranous and muscular VSDs, were found in several of the mice that died in the first day of life (n = 6/28 examined). Given the presence of outflow tract defects and VSDs, we examined whether the ductus arteriosus had closed appropriately or not at birth. Significantly, both Naa10−/− and Naa10+/− females (n=3/28 examined) exhibited a patent ductus arteriosus, meaning there is a failure of the mutant in utero cardiovascular system to adapt to adult life (birth) and close the interatrial and aorta-pulmonary trunk shunts that are required for normal fetal life (40). As murine outflow tract and VSD defects are not compatible with postnatal survival (40), these data suggest that congenital heart defects in Naa10−/− mice may explain some of their neonatal lethality (S2 Fig). We also examined surviving adult mice for any possible situs inversus, but we did not observe this in any adult (>4 weeks) Naa10−/− mice examined (n=19). Combined, these data suggest that Naa10 mutant CHDs are mainly confined to aberrant remodeling of the great vessels of the heart, leading to pulmonary overload at birth resulting in lethality.

Some of the surviving homozygous mice (Naa10−/− and Naa10+/−) had reduced body weight (Fig 2A). This reduced body weight continued through weaning, although some mice did lose weight as they developed progressive hydrocephaly. If the analysis was restricted to only include litters in which there was at least one Naa10−/− and one Naa10−/− mouse in the same litter alive to be weighed at P4 and beyond, it was consistently observed that the smallest weight animal among the two genotypes was almost always the Naa10−/− mice. For example, 13 litters met this criteria from the mating (Naa10+/− x Naa10−/−), and 12/13 of the litters had the Naa10−/− as the lower weight (Fisher's exact test, two-tailed, P value <0.0001). 5 litters met this criteria from the mating (Naa10+/− x Naa10+/−), and of these, all of them had the Naa10−/− as the lower weight (Fisher's exact test, two-tailed, P value = 0.0079). Therefore, despite the known variability in weight data as a function of genetic background, environment, and stochastic variation (41), it does appear at least for "within-litter" analysis that Naa10−/− males are born at a smaller weight than Naa10+/− males and remain the smallest male in the litter throughout their life.

Although piebaldism has never been reported in humans with OS, all (100%) of Naa10−/− and Naa10+/− mice exhibited hypopigmentation on their belly (Fig 2B, upper), with this piebaldism quite varied in its extent but not appearing to correlate in any way with other phenotypes, such as hydrocephaly. Another phenotype with complete penetrance was
bilateral supernumerary ribs (14 pairs of rib instead of 13) in all Naa10<sup>+/Y</sup> and Naa10<sup>m1a/Y</sup> mice (Fig 2B, middle and bottom, Table 1). This extra pair of ribs linking to the sternum transforms the T8 vertebrae into an anterior T7-like phenotype (S3A-S3D Fig, Table 1).

Fig 1. Deficiency of Naa10 leads to abnormal development and postnatal lethality. (A) Naa10<sup>+/Y</sup>, Naa10<sup>+/+</sup> and Naa10<sup>m1a/Y</sup> embryos at E10.5. Growth retardation (5/33, more than 5 somites lower or undersized compared to littermate controls), kinky trunk and developmental arrest are shown in Naa10<sup>−/−</sup> (4/33) and Naa10<sup>m1a/−</sup> (1/5). Scale bars: 500μm. (B) The percentage lethality in newborns, comparing Naa10 wild-type (Naa10<sup>+/+</sup> and Naa10<sup>+/Y</sup>), Naa10<sup>−/−</sup> and Naa10<sup>−/Y</sup> pups until P3, derived from matings between heterozygous females and wild-type (WT) males. Approximately 11.6% (10/86) of WT, 24% (13/54) of Naa10<sup>+/−</sup> and 76.3% (29/38) Naa10<sup>−/Y</sup> mice were found dead before P3. (C) Representative images of Naa10<sup>−/Y</sup> pups during early postnatal days compared with Naa10<sup>+/Y</sup>. Severe developmental defects such as malformations of head and lower body (one leg; black arrowheads), whole-body edema and anophthalmia (black arrows) are shown (N=1 each). (D) Hematoxylin and Eosin (H&E)-stained heart transverse section at E14.5 and vertical section at E18.5, comparing Naa10<sup>+/Y</sup> and Naa10<sup>−/Y</sup> embryos. Naa10<sup>−/Y</sup> embryo shows a VSD at E14.5 and E18.5. Also, at E18.5, Naa10<sup>−/Y</sup> embryo shows ASD. Arrow indicates VSD, ASD and DORV. Scale bars: 20 μm. VSD, ventricular septal defect; ASD, atrial septal defect; DORV, double outlet right ventricle.
Fig 2. Pleiotropic phenotypes of Naa10 KO mice. (A-C) Representative images of abnormalities in Naa10−/− compared with Naa10+/+.

(A) Body weight of male (left) and female (right) versus ages was monitored from 2 weeks. The weight of Naa10−/− and Naa10−/− mice is markedly reduced compared with that of the WT mice. Asterisks indicate a statistical difference calculated by Student’s t-test: *p < 0.05. (B) Representative images of completely penetrant phenotypes. Hypopigmentation (Naa10−/−, n=243; Naa10+/+, n=121; Naa10tm1a/Y, n=17) and supernumerary ribs (Naa10−/−, n=3; Naa10−/−, n=6; Naa10tm1a/Y, n=2; E18.5) were found 100% in Naa10 deficient mice. (C) Naa10−/− is smaller in size and has round-shaped head (Naa10−/− 0/59, Naa10−/− 7/33). Over time, hydrocephaly became apparent. (N=14/29 (~48%) for >P7 male Naa10−/−; N=7/19 (~36%) for >P7 female Naa10−/−).

Hydronephrosis (red arrow, Naa10−/− 0/23, Naa10−/− 14/29, Naa10−/− 0/5, Naa10−/− 7/19) and abnormal genitalia (black arrow) of male (middle, Naa10−/− 0/23, Naa10−/− 16/29) and female (bottom, hydrometrocolpos, Naa10−/− 0/5, Naa10−/− 7/19) are shown.
Table 1. Skeletal analyses for ribs, sternebrae, and vertebrae

<table>
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<tr>
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<th>Naa10⁺Y (n=50)</th>
<th>Naa10⁺⁺ (n=10)</th>
<th>Naa10⁻⁻ (n=17)</th>
<th>Naa10⁻⁻ (n=17)</th>
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<td>sternebrae</td>
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<td>3 sternebrae</td>
<td>7 (14.0%)</td>
<td>1 (10%)</td>
<td>3 (17.6%)</td>
<td>9 (52.9%)</td>
<td>1 (100%)</td>
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<td>4 sternebrae but with</td>
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<td>3/4 fusion</td>
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<td>14 ribs total bilaterally</td>
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<td>0 (0%)</td>
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<td>13 ribs total bilaterally</td>
<td></td>
<td>50 (100%)</td>
<td>10 (100%)</td>
<td>17 (100%)</td>
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<td>8 ribs attached to</td>
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<td>sternum bilaterally</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>17 (100%)</td>
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<td>7 ribs attached to</td>
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<td>sternum bilaterally</td>
<td>50 (100%)</td>
<td>10 (100%)</td>
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<tr>
<td>14 Thoracic vertebrae</td>
<td>0 (0%)</td>
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<td>13 Thoracic vertebrae</td>
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Tabulation regarding the number of sternebrae found in skeletons, including ones in which there was partial fusions between the 3rd and 4th sternebrae.

A majority of the Naa10⁺Y and Naa10⁻⁻ mice also had four instead of the usual three sternebrae, which were sometimes fused (Table 1). Cervical vertebrae fusion was also demonstrated in Naa10⁺Y mice, particularly involving C1 and C2, suggesting possible anteriorization of C2 into a C1-like phenotype (S3E and S3F Fig, and Table S3). The number of lumbar vertebrae remained the same, thus suggesting an anterior transformation of the first sacral vertebra to a lumbar-like phenotype. These combined observations suggest possible anterior transformations in the Naa10 mutant skeletal phenotype, with an anteriorization of C2, a T8 transformation to a T7-like phenotype with ribs connecting to the sternum, an extra pair of ribs on L1 likely due to an L1 transformation to a T13-like phenotype, and an anterior transformation of the first sacral vertebra to a lumbar-like phenotype with loss of fusion to the sacral wings.

Some Naa10 KO (Naa10⁺Y and Naa10⁻⁻) mice survived more than 4 months, with some of these then developing hydronephrosis (Fig 2C, middle). They had some hollowed space in the kidney, which had been filled with fluid and their ureter was thickened already at P3 stage of prenatal development in some Naa10⁻⁻ mice (S4A Fig). Commonly, hydronephrosis is caused by a blockage or obstruction in the urinary tract. We speculate that this swelling in Naa10 KO (Naa10⁺Y and Naa10⁻⁻) mice is likely caused by ureteral defects rather than the kidney itself. Moreover, some Naa10 KO mice displayed genital defects, such as seminal vesicle malformation and hydrometrocolpos, respectively (Fig 2C, bottom). Many Naa10⁻⁻ female mice appeared to have decreased fecundity, although they were fertile upon the first mating, and this decrease in fecundity is possibly due to the development of hydrometrocolpos (Fig 2C, bottom), which might result from structural issues, like vaginal atresia or a retained vaginal septum, although this requires further investigation. Additionally, hydrocephaly became clinically apparent with a round-shaped head (Fig 2C, upper) in some Naa10⁻⁻ mice. CT scanning of some of these mice confirmed hydrocephaly as the primary cause of their rapid deteriorating condition, usually within the first 3 months of life (S4B Fig). CT scanning did not reveal any obstructive lesions (such as a tumor) in any of the
ventricles that could account for the hydrocephaly. Taken together, these results indicate that
Naa10 contributes to overall development and is particularly important for viability.

Litter sizes and offspring from other matings were also investigated, as shown in
Table S4. Matings were set up between Naa10<sup>-/-</sup> females and C57bl6J WT (Naa10<sup>+/+</sup>) males,
involving eleven mating pairs with 7 unique females and 7 unique males. Of a total of 127
pups that were born, 37 died in the first day of life and were degraded and/or cannibalized
prior to any tail sample being retrieved, thus not being genotyped. This was a relatively high
death rate in the first 24 hours of life (29%), more so than with the other matings, except for
the one between Naa10<sup>-/-</sup> females and Naa10<sup>+/+</sup> males (Table S4). However, this is
substantially less than the death rate of 90% (46/51) reported for the same mating in the Lee
et al. paper (42), and we currently do not have an explanation for this discrepancy. Of the
remaining 90 pups that could be genotyped, 59 of these were Naa10<sup>-/-</sup> females and 31 were
Naa10<sup>+/+</sup> males. Seven of the 59 Naa10<sup>-/-</sup> females and two of the 31 Naa10<sup>+/+</sup> males died in
the first 3 days of life (for a total death rate in the first three days for all born pups of 46/127,
or 36%), and after this time, none of the remaining Naa10<sup>-/-</sup> females died in the first ten
weeks of life (88% overall survival), whereas ten of the 31 Naa10<sup>+/+</sup> males developed
hydrocephaly and died in the first ten weeks of life (61% overall survival). The death rate of
36% in the first three days of life is similar to the rate of 42.4% seen with the mating of
Naa10<sup>-/-</sup> females with Naa10<sup>+/+</sup> males (Table S4), whereas this rate is higher than that seen for
Naa10<sup>-/-</sup> females with Naa10<sup>+/+</sup> males (15.8%) and Naa10<sup>-/-</sup> females with Naa10<sup>+/+</sup> males
(13.6%).

**Naa10-deficient mice have a functionally active NatA complex**

OS shows reduced in vivo protein Nt-acetylation in patient cells (25). Reduced Nt-
acyetylomes were also observed in the Naa10 mutant yeast models (27). Given these prior
reports, we hypothesized that pleotropic phenotypes in Naa10-deficient mice are due to a
decrease in global N-terminal acetylation. To test our hypothesis, integrated N-terminal
peptide enrichment method (iNrich) (43) was used to analyze the level of protein Nt-
acetylation in mouse embryonic fibroblasts (MEFs) of Naa10<sup>+/+</sup> and Naa10<sup>+/+</sup>. To evaluate the
degree of Nt-acetylation, we applied a mass spectrometry (MS)-based N-terminomics method
to protein lysates from MEFs. Since the samples are treated with deuterated acetic anhydride
prior to MS, unacylated N-terminal site appears with +3 Da mass shift in the MS spectrum
of the corresponding acetylated N-terminal site (44). The peak intensity ratios of acetyl/heavy
acetyl pairs represent degree of acetylation of the N-terminal site. We found 765 acetyl/heavy
acetyl pairs of N-termini throughout five replicates of Naa10<sup>+/+</sup> and five replicates of Naa10<sup>+/+</sup>. Except for the sites detected only in either WT or mutant, 533 N-terminal sites could be
compared. Approximately 98% (n=523) of N-termini pairs showed less than 10% variation in
the degree of Nt-acetylation, indicating that there is no significant difference in Nt-acetylation
between Naa10<sup>+/+</sup> and Naa10<sup>+/+</sup> mice (Fig 3A). To further investigate this, we measured the in
vitro Nt-acetylation activity of NatA via immunoprecipitation of the large auxiliary subunit
Naa15 from mouse tissues. This analysis showed normal expression of Naa15 in Naa10 KO
liver tissue as in WT tissues (Fig 3B), and we isolated a physical complex composed of
Naa15 and undefined partners that retains NatA activity from Naa10 KO tissues (Fig 3C).
These data suggest that despite the loss of Naa10 in mice, the NatA complex remains active.
**Fig 3. Activity measurement of NatA from WT and Naa10 KO mice.** (A) Correlation of Naa10 alteration state on Nt-acetylation in mouse embryonic fibroblasts (MEFs). Each dot (n=533) represents the average Nt-acetylation percentage of 5 replicates of Naa10+/Y and Naa10−/Y, respectively. Dashed lines are the borders of ±10% difference. Except for the ten dots, 523 of the 533 dots are within the borders. The marginal histograms show the distribution of Nt-acetylation data points. (B) Immunoprecipitation of Naa15. Liver tissue from WT and Naa10 KO mouse was lysed and incubated with anti-Naa15 antibody to retrieve NatA complexes. Proteins were separated by SDS-PAGE and immunoblots probed with anti-Naa15 antibody and anti-NAA10 antibody. (C) Catalytic activity of immunoprecipitated NatA. The catalytic activity of NatA precipitated from WT and Naa10 KO mouse liver tissue by anti-Naa15 was measured towards the NatA substrate peptide SESS in an in vitro [14C]-Ac-CoA–Based Acetylation Assay. Control reactions were performed with no enzyme or no peptide to account for background signal. The immunoprecipitation (IP) and activity measurements were performed in three independent setups, each with three technical replicates per assay. One representative setup is shown.

**A Naa10 paralog exists in mice**

Naa10 disruption is lethal in a variety of organisms, including *D. melanogaster* (17), *C. elegans* (18) and *T. brucei* (45). Given the relatively mild phenotype and no reduction of the Nt-acetylome in Naa10 KO mice, we hypothesized that there might be a yet unidentified paralog of Naa10, which can compensate for loss of function in mice. A Blast search for genomic sequences with homology to Naa10 exposed several Naa10 pseudogenes on chromosome 2, 3, 7, 12, 15 and 18. Additionally, southern blot analysis from C57BL/6J DNA with Naa10 cDNA probe detected bands of the expected sizes on the X chromosome (S5A and S5B Fig), while other bands of unexpected sizes appeared on other chromosomes 2, 5, 15 and 18. The previously identified Naa10 paralog Naa11 is located on chromosome 5, however, this paralog is only expressed in testes (38). We identified a homologous sequence on chromosome 18, which we named Naa12. This predicted gene shows high similarity to Naa10, and RiboSeq and mRNA traces of this region suggest possible transcription and translation of this gene (S5C Fig). The protein sequence of Naa12 is >80% identical to Naa10. In addition, Naa12 is almost 90% identical with Naa11 (37).
Quantitative PCR (q-PCR) analysis also confirmed the expression of this transcript in all tested tissues (S6A Fig), with the expression of Naa12 unchanged in the corresponding Na10 KO tissues. We performed a sequence alignment of the two known mNaa10 isoforms, mNaa11 and mNaa12 and selected a unique Naa12 peptide for immunization and antibody generation (S6B Fig). After generation and affinity-purification, we validated the specificity and sensitivity of this Naa12 antibody (S6C and S6D Fig). Western blot analyses from mouse tissue demonstrated a band at around 35 kDa in both kidney and heart lysates, while we could not detect Naa12 in liver (S6E Fig). To assure that the antibody is specific, we also probed the membrane with Naa12 antibody that was blocked by the peptide it was raised against. The band at 35 kDa disappears when probed with the blocked antibody, which appears to confirm the specificity.

To test whether Naa12 has a similar enzymatic activity as Naa10, we performed a radioactive-based acetyltransferase assay using synthetic peptides (Fig 4A). Since monomeric Naa10 preferentially acetylates N-termini with acidic side chains (46-48), we used peptides representing the N-termini of γ-actin (starting DDDIA-) and γ-actin (starting EEEIA-), the two known Naa10 in vitro substrates. Additionally, we used a peptide starting with SESSSKS-, an in vitro NatA complex substrate High mobility group protein A1. As expected for the monomeric proteins, we could not detect any activity towards the SESSSKS- substrate. Importantly, both Naa10 and Naa12 significantly Nt-acetylated the acidic N-terminal peptides demonstrating the intrinsic capacity of Naa12 to catalyze Nt-acetylation (Fig 4A).

Across species, Naa10 is bound to its auxiliary subunit, Naa15, which links the catalytic subunit to the ribosome to facilitate co-translational Nt-acetylation of proteins as they emerge from the exit tunnel (23, 49-53). Due to its high sequence similarity (see also S4B Fig), we suspected that Naa12 may also interact with Naa15. To test this hypothesis, we performed co-immunoprecipitation assays in HEK 293 cells. Apart from Naa10 (isoform 1, Naa10235) and Naa12, we also included the second isoform of mNaa10, mNaa10225 that has been described earlier (12, 50, 54) as well as Naa11. Both Naa10 isoforms as well as Naa11 and Naa12 co-precipitated with V5-Naa15 but not V5 alone, suggesting that all tested proteins could form a stable complex with Naa15 in mouse (Fig 4B). In a mass spectrometry analysis of a similar setup to that shown in Fig 3B, immunoprecipitation of endogenous Naa15 from liver tissue derived from WT and Naa10 KO mice, we identified one peptide in the Naa10 KO sample that is identical between Naa12 and Naa11, but differing from Naa10 (Table 2). Further, we detected a number of peptides shared between Naa10, Naa11 and Naa12 in the Naa15 immunoprecipitates from the Naa10 KO sample. In contrast, peptides unique for Naa10 were only detected in the WT mouse sample. This together with data demonstrating that Naa11 is only expressed in testes and that no Naa10 is present in the Naa10 KO mice, strongly suggest that endogenous Naa15-Naa12 complexes are present in Naa10 KO mice.
**Fig 4. Characterization of Naa12.** (A) In vitro N-terminal acetyltransferase radioactive-based assay. Comparison of mouse Naa10 and Naa12 towards Naa10 peptide substrates, beta-actin (DDDIA-) and gamma-actin (EEEIA-), and the optimal NatA complex peptide substrate, SESSS-. Background control reactions were performed in the absence of either peptide or enzyme. Assays were performed in triplicate; error bars represent S.E.M. (B) Co-immunoprecipitation assay. HEK293 cells were transfected as indicated and lysed after 48 h. Cell lysates were incubated with 1 μg anti-V5 antibody to precipitate V5-tagged Naa15. The isolated complexes were separated on SDS-PAGE and probed with the indicated antibodies.
Table 2. Naa10, Naa11 and Naa12 peptides identified by LC-MS/MS analysis in Naa15 IP samples from WT and Naa10 KO mouse

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Peptide sequence</th>
<th>Log 2 intensity</th>
</tr>
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<tbody>
<tr>
<td>Naa11/Naa12</td>
<td>AALHLYSNTLNFLQVSEVEPK</td>
<td>23,4375</td>
</tr>
<tr>
<td>Naa10/Naa11/Naa12</td>
<td>IVGYVLAK</td>
<td>27,0429</td>
</tr>
<tr>
<td>Naa10/Naa11/Naa12</td>
<td>MEEDPDDVPHGHTSLAVK</td>
<td>27,7220</td>
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<tr>
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<td>MEEDPDDVPHGHTSLAVKR</td>
<td>24,5443</td>
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<tr>
<td>Naa10/Naa11/Naa12</td>
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<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

*Samples were run in technical duplicates and the average log2 intensity of the peptides are presented.

Naa12 may rescue loss of Naa10 in mice

To investigate whether Naa12 can rescue the loss of the function of Naa10 in vivo, Naa12 KO mice were generated using CRISPR technology (55). One 95-base pair deletion Δ131-225 in Naa12 was characterized in depth (Fig 5A). This mutation introduces a frameshift, leading to a termination codon at amino acid 67, which should either result in complete knockout of the protein or, at best, the expression of a truncated mini-protein that would be far shorter than the usual 220 amino acid Naa12. We confirmed the deletion by PCR with genomic DNA (Fig 5B). QPCR further showed deletion of Naa12 in the tested tissues of Naa12 KO mice (Fig 5C), however, it seemed that Naa12 might be slightly expressed in testis. Due to the high similarity between Naa11 and Naa12, the expression shown in Naa12 KO testis could actually be Naa11 rather than Naa12, and this was confirmed by RT-PCR showing definite deletion (S7A Fig). This was confirmed at the protein level with the Naa12 antibody in heart tissue lysates (S7B Fig). However, this antibody was unable to consistently detect protein even in WT liver, kidney, or brain tissue lysates, which could be due to very low expression or post-translational modification of Naa12 in these tissues. Furthermore, given that this antibody is raised against a peptide at the C-terminus of Naa12, these results do not completely exclude the possibility of truncated non-functional mini-protein expression, although the lack of any signal with RT-PCR (S7A Fig) likely means that nonsense-mediated decay occurred.
Fig 5. Generation of Naa12 KO mice. (A) Scheme of Naa12 deletion used to generate Naa12 KO mouse. 95 base pairs (131-225) were deleted. F': genomic DNA forward primer, F'': cDNA forward primer, R; reverse primer. (B) Genotyping of Naa12 KO mice by PCR. WT allele size was 381bp and targeted allele size was 287bp. (C) mRNA level of Naa12 were analyzed in selected tissues by qPCR. Relative expression level of WT (white bars) and Naa12 KO (black bars) after normalizing to that of GAPDH.

Homologs that play a compensatory role usually show similar phenotypes to each other when one of them is deficient (56). Therefore, we analyzed Naa12 KO mice to see if they produced similar developmental defects to those in Naa10 KO mice. Unexpectedly, knockout mice for this gene were viable (Table S5), albeit at a reduced rate of fertility (Table S4). Necropsy and inspection of testes and seminal vesicles under a stereomicroscope, along with weighing these tissues, did not reveal any macroscopic differences that could readily explain the decreased fertility of the Naa12 KO males. Furthermore, the phenotypes (piebaldism and bilateral supernumerary ribs; Fig.2b) observed in Naa10 KO mice with complete penetrance were not present in Naa12 KO mice (S7C Fig). Overall, there were not any obvious phenotypes in these mice, except for reduced fertility.

Matings between compound heterozygous (Naa10+/--; Naa12+/--) females and Naa10+/Y; Naa12+/-- males demonstrate that no live births occurred for Naa10; Naa12 double-knockout (DKO) males (Naa10+/Y; Naa12--/--) (Fig 6A). In addition, the average litter size was
very small when compared to the control (WT x WT) matings, suggesting embryonic lethality (Table 3 and Table S4). In order to determine whether lethality occurs during the embryonic stage, we genotyped E18.5 litters – just before birth. Consistent with our previous observations, we could not obtain any Naa10\(^{+/+}\); Naa12\(^{-/-}\) embryos, and many embryos could not be genotyped because they were already in the midst of resorption (n=23) (Fig 6). We checked an even earlier stage at E10.5 and also found zero Naa10\(^{+/+}\); Naa12\(^{-/-}\) embryos, and also with far fewer resorptions at this stage (N=3). Interestingly, we did observe Naa10\(^{+/+}\); Naa12\(^{+/+}\) embryos where two of them displayed delayed developmental stage (appearing younger than E10.5) and another two embryos were lysed and had already begun degenerating (but despite this, we could at least genotype these embryos). This helps explain why only one Naa10\(^{+/+}\); Naa12\(^{+/+}\) embryo was observed at E18.5. Furthermore, Naa10\(^{+/+}\); Naa12\(^{-/-}\) female embryos were also lysed/degenerating at E10.5 and were not observed from that day onward. Matings between compound heterozygous females and Naa10\(^{+/+}\); Naa12\(^{-/-}\) males also did not yield Naa10\(^{+/+}\); Naa12\(^{-/-}\) male or Naa10\(^{+/+}\); Naa12\(^{-/-}\) female mice during development (S8 Fig). Consistent with our previous findings, we noted many resorptions at E12.5 and E18.5 that could not be genotyped. The number of living postnatal compound heterozygous female mice was also considerably lower than the predicted Mendelian ratios (Fig. 6 and S8 Fig), and the surviving Naa10\(^{+/+}\); Naa12\(^{+/+}\) females were smaller in size than littermate controls (Fig. 7D). Due to the severe embryonic lethality observed in the Naa10; Naa12 DKO male mice and the Naa10\(^{+/+}\); Naa12\(^{-/-}\) female mice, which was not seen in each single KO (Naa10 KO or Naa12 KO), it seems likely that, without compensation by Naa12, NTA is disrupted in Naa10; Naa12 DKO mice. Together, these data support the compensatory role of Naa12 in vivo.

### Fig 6. Lethality in Naa10; Naa12 DKO mice.

(A) Naa10; Naa12 DKO exhibit embryonic lethality. Pedigree and genotypes of pups and embryos at E10.5 and E18.5 from Naa10\(^{+/+}\); Naa12\(^{+/+}\) female mice crossed to the Naa10\(^{+/+}\); Naa12\(^{-/-}\) male mouse.
Table 3. Litter size of Naa10;Naa12 DKO matings

<table>
<thead>
<tr>
<th>Genotypes of Naa10; Naa12 breeders (♀ x ♂)</th>
<th>Total number of pups</th>
<th>Total number of litters</th>
<th>Average litter size (pups/litters)</th>
<th>SD of litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naa10&lt;sup&gt;+/+&lt;/sup&gt;; Naa12&lt;sup&gt;+/+&lt;/sup&gt; x Naa10&lt;sup&gt;+/+&lt;/sup&gt;; Naa12&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>206</td>
<td>24</td>
<td>8.6</td>
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</tr>
<tr>
<td>Naa10&lt;sup&gt;+/+&lt;/sup&gt;; Naa12&lt;sup&gt;+/+&lt;/sup&gt; x Naa10&lt;sup&gt;+/Y&lt;/sup&gt;; Naa12&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>157</td>
<td>32</td>
<td>4.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

SD : Standard Deviation

Statistical examination of weight data in Naa10- and Naa12-deficient mice

To determine whether Naa10 and Naa12 are essential for viability and development, we examined the survival, weights, and growth rates of 688 Naa10 and Naa12 knockout and wild type mice. The genotypes of mice examined are listed in Table S6. To avoid potential survival biases, only weights taken during the first 180 days were included. Growth curves are shown in Fig 7. Age and age-squared (the quadratic term) are both entered in the analyses; the quadratic term shows the degree to which the effect of age itself changes with age.

Table S7 shows the results in which weight of Naa10 mice in grams is regressed upon age, Naa10 knockout status, and their interaction. Unsurprisingly, age predicts weight for males and females strongly, with growth slowing with age (first column). Though a strong negative effect of the knockout is seen in for both males and females (second column), when both age and knockout status are modeled together (third column) this effect all but disappears in females. Moreover, in females there is no interaction of knockout status with age (fourth column), suggesting that the Naa10 knockout status itself has no significant effect on the growth rate in females. For males, however, the main effect of the knockout remains when age is included in the model (third column) and the interaction is significant (fourth column), indicating that the Naa10 knockout both reduces weight of males overall and lowers the rate of growth.

Results of analyses of mixed-genetic background Naa10/Naa12 mice are shown in Table S8. Effects of age and knockouts on weight comprise the upper portion of the table, while the lower portion shows their effect on the rate of weight gain. Among females a significant reduction of weight (above, second column) and in the rate of growth (below, first column) is seen among mice heterozygous for the Naa10 KO. There were no homozygous Naa10 KO female mixed-breed mice available to analyze, as the matings were not set up to yield any such mice (so breeding patterns, not mortality in utero, are the reason for this absence). No significant effect on growth rate is seen for heterozygous or homozygous Naa12 KO (above, third column) or for their interactions with age (below, second column), and only the effects of the heterozygous Naa10 KO and its interaction with age are seen in the full model (below, third column). Thus the Naa12 KO, whether heterozygous or homozygous, does not appear to reduce the weight or growth rate of females, while a heterozygous Naa10 KO is sufficient to reduce both weight and growth rate. Interestingly, when modeled together, both the Naa10 and the Naa12 KOs significantly reduced weight (above, fourth column) and the interaction of the Naa10 and the Naa12 heterozygous KOs significantly reduced weight (above, fifth column). As no female mice were both KO for Naa10 and homozygous KO for Naa12, the effect of the interaction of those two factors could not be determined. The triple interaction of heterozygous Naa10 KO, Naa12 KO and age was weakly significant, suggesting that the presence of both knockouts affects growth rate above and beyond the effects of each knockout independently (below, fourth column). No males with knockouts of both Naa10 and Naa12 were born, so no test of their interaction was possible. An effect was seen for the Naa10 knockout on weight when modeled with age and age² (second column), and the significant interaction of the Naa10 knockout with age and age² (third column) shows that the Naa10 KO in males reduces the growth rate. As with females, no significant effect of a Naa12 KO, whether heterozygous or homozygous, was seen in males, nor is there a significant interaction with age (fourth column). When the interactions of age with both Naa10 and Naa12 knockout status are entered in one model, Naa10 alone is seen to reduce growth rates (fifth column).

**Discussion**

We have shown that Naa10 deficiency results in pleotropic developmental defects in two different Naa10-deficient mouse models. Similar to infant mortality in some OS males, the lethality of Naa10 KO mice increased dramatically in pups in the first 3 days of life (Fig 1B). Defects in kidney, brain, pigmentation (piebaldism), and ribs were observed during embryonic or early postnatal stages in some mice (Fig 2B and 2C). These observed
phenotypes overlap with some of the phenotypes found in surviving humans with OS, including supernumerary vertebrae and hydrocephaly, although piebaldism has not been reported to date in any humans. However, the puzzling lack of embryonic lethality in the Naa10 KO mice prompted us to discover Naa12 as a possible compensatory NAT, with Naa10-like NTA activity (Fig 4A), with an interaction between Naa15 and Naa12 (Fig 4B). In addition, co-immunoprecipitation of endogenous Naa15 from Naa10 KO mouse tissues, followed by mass spectrometry analysis (Table 2) and Nt-acetylation assays (Fig 3C) fully support that the endogenous Naa12-Naa15 complexes produces NatA activity. Finally, we found genetic proof of the compensatory activity of Naa12 in mice when we observed embryonic lethality in in Naa10; Naa12 DKO male and Naa10+/--; Naa12−/− female mice (Fig 6 and S7 Fig). This compensation by Naa12 explains the mouse proteomics data indicating normal Nt-acetylation in Naa10 KO mice (Fig 3A). We have confirmed the expression of Naa12 in various tissues using qPCR and Western blot analyses (S5A-S5E Fig). The band for Naa12 runs a little higher in the Western blot than the calculated molecular weight would suggest, which is consistent with previous observations of NAA10 gel migration. Future characterization of Naa12 may define possible post-translational modifications specific for Naa12 that might account for the variability of detection by the antibody.

Gene duplication has long been believed to be a major driving force in evolution that provides genetic novelty in organisms. Paralogous genes, originating by small-scale or whole-genome duplication, overlap functional roles for each other and can completely or partially compensate for the loss of the duplicate gene (56, 57). There is not yet any human reported with complete knockout for NAA10. There is one published truncating variant in the C-terminal portion of NAA10 in a male patient with microphthalmia (35), but unfortunately there are no cell lines available from this family to confirm whether any truncated NAA10 protein is expressed, as was shown with a splice-site mutation in a Lenz microphthalmia family (29). NAA10 was also identified in screens for essential genes in human cell lines (58, 59), so it seems unlikely that an unknown NAA10-like paralogous gene exists in humans, other than the already known NAA11.

The pleiotropic phenotypes shown in Naa10 KO mice, including hypopigmentation and supernumerary ribs with a penetrance of 100%, were not observed in the Naa12 KO mice. Naa10 itself has been described to have N-e-acetyl-activity towards internal lysine residues of proteins involved in various disease- and development-related signaling pathways (11), although its acetylation of some substrates is controversial (60, 61). Since the Nt-acetylome appears to be globally intact in MEFs from Naa10 KO mice, it is possible that the presented phenotypes could be due to the loss of Naa10-specific N-e-acetyl-activity or non-catalytic roles of Naa10 (4). Alternatively, the quantitative expression of Naa10 and Naa12 might be different within or between tissues, which might then explain why there is clearly a phenotype for Naa10+/--; Naa12−/− female mice (not born at Mendelian ratios and the few that are born are usually much smaller) but no apparent phenotype in Naa10+/--; Naa12−/− female mice. It seems likely that the mechanism cannot be simply additive between two equally expressed proteins, because if the expression of each protein is theoretically set at an arbitrary unit of 10, then Naa10+/--; Naa12+/− female mice might possibly have half as much of each protein, so that the total dose of both proteins together would be 10, instead of 20. Likewise, the total dose of both proteins together would also be predicted to be 10 in a Naa10+/--; Naa12−/− female. Yet, the Naa10+/--; Naa12+/− female mice have a phenotype, whereas the Naa10+/++; Naa12−/− female mice do not (Fig 6). Therefore, other explanations could include different tissue-specific dosages of each protein, different expression between different tissues, possible X-chromosome skewing for the X-linked Naa10 in different tissues, or
different functions of the two enzymes, including Naa10-specific N-e-acetyl-activity or non-catalytic roles of Naa10 (4). These questions remain unanswered and are worth exploring in future studies.

There are several clinical features that were presented in the original description of OS (26) which can now be better understood in light of the phenotypes found in the knockout mouse model. For example, all of the affected children in the first families with OS were noted to have large and, in some cases, persistently open fontanels (25, 26). For one child (Family 1, Individual II-1), CT scanning revealed cerebral atrophy with enlarged ventricles, and in another child (Family 1, Individual III-4), there was evidence on magnetic resonance imaging (MRI) of "moderate lateral and third ventricular dilatation without identified cause". Lastly, all of the children had respiratory depression and apneic episodes, along with varying course of hypotonia and/or hypertonia (including documented hyperreflexia in at least one case (Family 2, Individual III-2)). In retrospect, it seems that these clinical features could be consistent with mild hydrocephaly in these probands with Ogden syndrome, which resolved over time. This is also consistent with the ventriculomegaly reported in several female OS probands with missense mutations in NAA10, along with ventriculomegaly in one other male proband who died in the first week of life, with generalized hypotonia and lack of spontaneous respirations (62). One of the female patients with an Arg83Cys mutation in Naa10 (#9 in Table 1 of that paper) was reported as having intraventricular hemorrhage in the occipital horn, hypoxic-ischemic encephalopathy, and a ventriculo-peritoneal shunt. It is possible that this sequence of events is compatible with hydrocephaly with clinical signs and symptoms that required the placement of the shunt.

There are additional cardiac and skeletal features that are also worth re-examining in light of these new findings. In some of the original cases of OS, there were varying levels of pulmonary valve stenosis detected on echocardiography, along with some documentation of pulmonary hypoplasia (26). For example, individual III-7 in Family 1 was found on echocardiography to have small persistent ductus arteriosus, a mildly decreased left ventricular systolic function, an abnormal appearing aortic valve, an enlargement of the right ventricle, decreased right ventricular systolic function, and persistence of the foramen ovale. Individual III-6 from this same extended family was found on echocardiography to have a thickened bicuspid aortic valve and mild pulmonary hypertension. One of the OS female patients with an Arg83Cys mutation in NAA10 was reported to have "supernumerary vertebrae" (62). Prompted by our findings of supernumerary ribs in the mice, we obtained an MRI report for this patient, in which the radiologist concluded that there appeared to be 25 distinct vertebrae, as opposed to the usual 24, with a suggestion of a 13th rib, at least on the right. The report went on to state that "the vertebrae represent 7 cervical vertebrae, 13 rib-bearing thoracic vertebrae, and 5 lumbar vertebrae, and the L1 vertebra is mildly dysmorphic, with a suggestion of anterior beaking". In addition, chest and abdominal X-rays from two of the brothers in generation VI of a family with microphthalmia demonstrated the presence of 13 rib-bearing thoracic vertebrae, alongside the dramatic scoliosis in both individuals. Four other females carrying mutations in Naa10 were reported as having either pectus carinatum or excavatum (62), one of the boys with OS (Family 1, Individual III-4) was noted to have pectus excavatum, and retrospective review of some of the clinical photographs appears to show mild pectus excavatum in individual III-6 of the same family. Studies of human populations have shown that the levels of transition may be shifted cephalad, resulting in 23 mobile vertebrae, or shifted caudad, resulting in 25 presacral vertebrae. Such variations may occur in 2-11% of the population (63). In addition, the number of ribs can also vary in mice, as a result of teratogenic and genetic influences (64, 65). However, the complete penetrance
for supernumerary ribs in the Naa10-deficient mice, along with the presence of extras ribs in some of the patients, suggest that there is a pathway common to humans and mice that is altered by mutations involving NAA10.

Several mouse mutants show similar cardiac or skeletal phenotypes to the Naa10-deficient mice. Pax3 mutants phenocopy our Naa10<sup>−/−</sup> mutants, as Pax3<sup>+/−</sup> adults exhibit 100% piebaldism, and exhibit neural crest (NC)-related PTA/DORV with concomitant VSDs (66-69). Pax3 systemic nulls also have skeletal defects due to abnormal somite morphogenesis (70, 71). Moreover, Pax3 cKOs demonstrated that NC-specific deletion is sufficient to cause DORV/VSDs and death at birth (69, 72), and that restricted deletion within the neuroepithelium causes congenital hydrocephalus (73). While Pax7 systemic deletion does not cause NC-associated defects, it does exhibit overlapping expression, and Pax3-Pax7 compound heterozygous mice develop hydrocephalus (73), suggesting combinatorial function. Hox C8<sup>−/−</sup> mice exhibit an extra rib and an extra rib articulating with the sternum (74, 75), and an unfused sacral vertebra which lead to 27 presacral vertebrae (76), as seen in our model. Hox A4<sup>+/−</sup> mice described in Horan et al. (77) shows cervical fusions of C2/C3, a rib on C7 not fully penetrant and sternal defects with bone ossification anomalies. Hox A5<sup>−/−</sup> mice display numerous cervico-thoracic defects such as a rib process coming from the 7th cervical vertebra, an increased in the number of sternbrae and total number of ribs (78). Both Hox A4<sup>−/−</sup> and A5<sup>−/−</sup> mice exhibit an extra rib articulating with the sternum. Hox D3<sup>−/−</sup> mice are the only Hox gene mutation leading to cervical fusion of both the atlas and axis (79). Hox A9<sup>−/−</sup> mice have anteriorization of both sacral and lumbar parts, with an extra pair of ribs at the lumbar level. Hox A9<sup>−/−</sup> mice do not have any relevant sternal defect (80). Hox B9<sup>−/−</sup> mice have an extra rib articulating with the sternum and 14 pairs of rib (81). These phenotypes, especially Hox C8, share common features with the Naa10-deficient mice. This phenotype is also close to the Rpl38<sup>−/−</sup> phenotype (82), except for the sacral fusion described in Rpl38<sup>−/−</sup> mice. Interestingly, it was shown that Hox genes were dysregulated in this genotype. The skeletal findings and comparison to other mutant mice suggest a pattern consistent with a homeotic anterior transformation hypothesis.

The developmental role of Naa10 in mice has been previously described (42). Lee et al. reported embryonic lethality at E12.5-14.5 and beyond (due to placental defects), hydrocephaly, postnatal growth retardation, and maternal effect lethality in Naa10 KO mice and suggested that genomic imprinting dysregulation is associated with those developmental phenotypes. In the present study, hydrocephaly and postnatal growth retardation were also apparent, but embryonic lethality was not observed, which prompted the search for and discovery of Naa12. The previous paper (42) did not report the piebaldism, homeotic anterior transformation, hydromephrosis, and genital defects (such as seminal vesicle malformation and hydrometrocolpos), nor did they explain the cause of death in the first day of life, which is at least partly due to congenital heart defects, as reported herein. A more recent paper from the same group reported that conventional and adipose-specific Naa10p deletions in mice resulted in increased energy expenditure, thermogenesis, and beige adipocyte differentiation in the surviving mice (83). Although the Lee et al. paper reported a very high maternal effect lethality rate of 90% (46/51) (otherwise stated as a survival rate of 10% (5/51)) for newborns in matings following Naa10<sup>−/−</sup> female and C57BL/6J wild type male intercrossing, this rate was only 29% (37/127) in this same mating herein in the first 24 hours of life and with a total death rate in the first three days for all newborns of 46/127, or 36% (Table S4), with this result deriving from a larger number of mating pairs, litters and pups. Although this rate of 36% is higher than that seen with matings involving Naa10<sup>−/−</sup> females (15.8% and 13.6%) (Table S4), the explanation for this ~20% difference in survival in the first 3 days of life
could involve differences in maternal care provided by the Naa10\textsuperscript{+/−} and Naa10\textsuperscript{−/−} females, but this would have to be investigated in future studies, involving detailed behavioral and cognitive assessment of the dams.

The reasons for the differences between the studies in regards to maternal effect lethality and in utero lethality are unknown at present. Whilst Lee \textit{et al.} deleted Naa10 exons 2-6 (42), the current study deleted Naa10 exons 1-4 or used an allele Naa10\textsuperscript{tm1a} expressing β-galactosidase instead of the Naa10 gene (S1 Fig.), and there was not any significant embryonic lethality in either line (S1 and S2 Table). All three of these mouse models were made using 129Sv/Ev ES cells, and all three are nulls lacking Naa10 protein. It is the case that the previous study used the Cre/loxP system to generate the Naa10 KO mice, where a floxed Naa10 female mouse was crossed with the Ella-Cre transgenic male mouse expressing Cre recombinase for germ line deletion of loxP-flanked Naa10, whereas our mice were made using standard gene-targeting methods without the use of Cre recombinase, but it is not clear how this would have resulted in embryonic lethality, particularly as these mice were only used after "at least six generations of backcross with C57BL/6 mice". The explanation for differences in embryonic lethality might be more likely due to different combinations of modifying alleles that are present in the different C57BL/6J genetic backgrounds, rather than differences in our model systems, and future plans will address this after back-crossing more than 20 generations to C57BL/6J to achieve an entirely inbred line. The impact of genetic background is supported by the observation that additional null alleles on mixed genetic backgrounds, made during the process of generating missense mouse models for OS, have far less penetrance for a range of the various phenotypes, including much less perinatal lethality (unpublished observations).

In conclusion, our study provides strong evidence that Naa10, the catalytic subunit of N-acetyltransferase A (NatA), is critical for normal development in mice. Furthermore, this study explains the puzzle regarding the lack of complete embryonic lethality in the Naa10 knockout mice due to the discovery of a second mouse Naa10 paralog, which, unlike Naa11, is expressed in the heart as well as other tissues. Taken together, our findings suggest that the newly identified Naa12 can functionally rescue Naa10 loss and act as a catalytic subunit in mouse NatA complexes.

Materials and Methods

Mice. All experiments were performed in accordance with guidelines of International Animal Care and Use Committee (IACUC) of Ewha Womans University, Cold Spring Harbor Laboratory, and Institute for Basic Research in Developmental Disabilities (IBR).

Generation of Naa10 deficient mice. The Naa10 knockout (KO) mice were generated as previously described (39). Naa10\textsuperscript{tm1a} B6;129P2-Ard1\textsuperscript{tm1a(Eucomm)Gio/J} (Naa10\textsuperscript{tm1a}) mice, used for Naa10 reporter mouse, were generated using standard method based on a standard gene-targeting in E14 embryonic stem (ES) cells (129/Sv) by using a targeting vector from EUCOMM. Correctly targeted ES clones were used for blastocyst microinjection and generation of chimeric mice. Chimeric mice were crossed to C57BL/6J mice, and then the progeny were backcrossed to C57BL/6J for more than ten generations. The Naa10-deficient mice used in the weight analyses were derived from mice backcrossed 8 times to a C57BL/6J inbred genetic background, and this was confirmed with genome scanning at the Jackson Laboratory, showing heterozygosity for only one marker for 129S1/SvImJ out of 290
autosomal markers tested, thus giving a percentage of C57BL/6J of 99.66%.

**Generation of Naa12 knockout mice.** The mice were made using standard methods by microinjection of CRISPR reagent mix into zygotes obtained from the mating of B6D2F1 females (i.e. 50% C57BL/6J, 50% DBA/2J (D2)) females to inbred C57BL/6J males. The guide RNA was produced and validated from Sigma using a Cell1-nuclease assay, and the most active guide was selected, which was Naa12_0_125 (C9587), with a target sequence of: GAGCGTTTCACAGCCAGCG and including the targeting cr-RNA sequence and the tracrRNA portion. The indels were transmitted by breeding again to inbred C57BL/6J males, and the resulting progeny were interbred on a mixed genetic background of approximately 12.5% DBA/2J (D2) / 87.5% C57BL/6J, for use in the reported experiments, including the weight analyses. Progeny from these mice have been backcrossed to C57BL/6J for more than ten generations, with no discernible new phenotypes emerging. Genomic DNA was isolated from paw and tail. DNA was screened for mutations using PCR and Surveyor assay (84), followed by Sanger sequencing of selected clones and the use of CRISP-ID (85) to identify putative deletions.

**Primers for mice genotyping.** The primers used for Naa10 KO and Naa10*tm1a* genotyping were Naa10-F: 5'-ectcactaatgctgtgcaa-3', Naa10-neo-F: 5’-acgcgtacctatgactcg-3’, Naa10-R: 5’-tgaaatcggtgcttgaga-3’, Naa10*tm1a*-F: 5’-gcacctctctactggac-3’, Naa10*tm1a*-neo-F: 5’-gcggctctttctgcatc-3’ and Naa10*tm1a*-R: 5’-cagggaaaagctggg-3’. The primers used for Naa12 KO were Naa12 Surveyor F: 5’-gtcctacgctgtaactgg-3’, Naa12 Surveyor R: 5’-gccagatgtcgatgacatgc-3’ and HEX-Naa12 Surveyor F: 5’-gtcctacgctgtaactgg-3’.

**Antibodies.** The following antibodies were used: Rabbit anti-Naa10 (Abcam #ab155687), rabbit anti-Naa10 (Protein Tech #14803-1-AP), rabbit monoclonal anti-NAA10 (CellSignaling, #13357), goat anti-Naa10 (Santa Cruz, #sc-33256), rabbit anti-Naa10 (Santa Cruz, #sc-33820), rabbit anti-Naa11 (Novus Biologicals; #NB1-90853), mouse anti-Naa15/NARG1 (Abcam; #ab60065), rabbit polyclonal anti-NAA15 (12), rabbit anti-Naa50 (LifeSpan BioSciences; #LS-C81324-100), rabbit anti-FLAG (Sigma; #F7425), mouse anti-GAPDH (Abcam; #ab9484), goat anti-Actin (Santa Cruz, #1615), mouse anti-GST (GenScript; #A00865) and mouse anti-V5 (Life Technologies; #R960-25). The antibody against the potential mNaa10 paralog mNaa12 (Gm16286, UniProt: Q9CQX6) was raised in rabbits after immunization with a synthetic peptide of the Naa12 C-terminus (aa191-215: QENLAGGDSGSDGKD-C) by PrimmBiotech.

**Alcian blue and Alizarin red co-staining of skeletons.** After the skin and internal organs were removed, embryos were fixed in 95% ethanol (EtOH) for 4h, then in 100% acetone for overnight. Embryos were stained with 0.03% Alcian Blue 8GX in ethanol/ acetic acid (4:1 v/v) for overnight and kept in 1% KOH for 2 days until they became clearly visible, followed by staining with 0.05% Alizarin Red in 1% KOH for 4h. After washing with 100% Glycerol/ 1% KOH (1:1 v/v), skeletons were kept in 100% Glycerol.

**Isolation and imaging of mouse embryos.** Timed mating were performed either by using the presence of a vaginal plug to assess fertilization. The morning vaginal plug was designated E0.5. Pregnant mice were sacrificed at several time points after conception. The embryos were isolated in ice-cold PBS with 1% FBS and washed three times in ice-cold PBS. Embryos were imaged using a Zeiss Axiozoom V16 with Zen software and merged 50 slides between Z-stack intervals.
β-galactosidase staining. Isolated E10.5 embryos were incubated in fixation solution (4% paraformaldehyde) at 4°C for 25 min. Samples were washed in ice-cold PBS and then incubated in permeabilization solution (PBS containing 0.01% Na deoxycholate, 0.02% Nonidet-P40, 2 mM MgCl2) for 20 min at 4°C. Subsequently, samples were incubated in β-gal staining solution (PBS containing 1 mg/mL X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.02% Nonidet-P40, 2 mM MgCl2) at 37°C overnight. Following β-gal staining, samples were washed with PBS and incubated in fixation solution at 4°C for storage.

Hematoxylin and Eosin Staining. Isolated kidney tissues at E18.5 and P3 were fixed with 4% paraformaldehyde at 4°C for overnight and embedded in paraffin. Samples were sectioned at 8 micron thick and stained with Hematoxylin (MHS80, Sigma) and Eosin (HT110116, Sigma) for morphology.

Cloning. Full-length mouse Naa10 and Naa12 expression vectors were separately constructed using a pMAL-c5x vector. In both cases, the catalytic subunit contained an N-terminal uncleavable MBP-tag. Bacterial expression vectors of mNATs were cloned from cDNA generated from mouse liver or testes. mRNA was isolated using the Oligotex direct mRNA kit (Qiagen) according to the manufacturer recommendations. 1µg RNA was reverse transcribed with Superscript IV reverse transcriptase (Thermo Fisher) and Oligo dT(18) primer. The PCR product was digested and cloned into BamHI restriction sites of pGEX-4T1 (GE Healthcare), pMAL-p5X and p3xFLAG-CMV10 (Sigma-Aldrich) using standard techniques. All constructs were sequenced to validate correct insert and orientation.

Primers for cloning. cDNA was amplified using the primers CCG GGA TCC ATG AAC ATC CC CG AAT CTG GGA TCC CTA GGA GGC AGA GTC AGA for mNaa10 variants, CCG GGA TCC ATG AAC ATC CGC AAT GC and CTG GGA TCC CTA GGA GAT GGA ATC CAA GTC for mNaa11, CCG GGA TCC ATG AAC ATC CGC CGG and CTG GGA TCC CTA GGA GGC GGA CCC TAG for mNaa12.

Peptide competition assay. To determine the specificity of the mNaa12 antibody, a peptide competition assay was performed using the same peptide as used for immunization (aa 191-205: QENLAGGDSGSDGKD-C). 100 µg antibody were bound to 50 mg peptide-coupled CNBr-sepharose (10 mg peptide/g sepharose) in PBS + 0.2 % Triton X-100 for 1 h at 4°C on an orbital shaker. The beads were pelleted by centrifugation at 2,700 × g for 3 min at 4°C and 250 µl of the antibody-depleted supernatant diluted in 5 mL TST for detection (1:100 final antibody dilution). Western blots of mouse lysates were probed with the depleted antibody or untreated antibody as control (1:100 dilution in TST).

Co-immunoprecipitation assay. Protein-protein interaction studies were performed in HEK293 cells. Briefly, 8 x 10⁵ cells were seeded per well in 6-well plates. After 24 h, cells were co-transfected with pcDNA3.1/V5-His-mNaa15 and p3xFLAG-CMV10-Naa10(235 isoform 1), -Naa10(225) (isoform 2), -Naa11 or -Naa12 or the corresponding empty vectors. Cells were lysed after 48 h in 200 µl PBS-X per well and cellular debris pelleted at 20,800×g for 10 min at 4°C. 350 µl of the generated lysate was incubated with 1 µg anti-V5 antibody for 1 h at 4°C, followed by a 30 min incubation with 30 µl protein-A sepharose (Sigma-Aldrich). Protein complexes were washed three times by centrifugation (2,700×g, 2 min) and eluted in 30 µl 2×SDS sample buffer.
Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Protran 0.2 μM NC) by immunoblotting. The membrane was blocked in 5% non-fat dry milk and incubated overnight with rabbit polyclonal anti-Naa15(12) (1:2000, BioGenes) and rabbit monoclonal anti-Naa10 (anti-ARD1A, 1:1000, CellSignaling, #13357) diluted in 1×PBS containing 1% non-fat dry milk and 0.1% Tween. The immunoblots were washed and incubated for 1 h at RT with HRP-linked secondary antibody donkey anti-rabbit IgG (GE Healthcare, NA934). The HRP-signal was detected using SuperSignal West Pico PLUS Chemiluminescent Substrate Kit (Thermo Scientific) and ChemiDoc XRS+ system (Bio-Rad) and visualised by ImagelabTM Software (Bio-Rad).

**Immunoprecipitation of Naa15 to form NatA complex.** For immunoprecipitation of Naa15, 90-120 mg liver tissue from a WT- and Naa10 KO mouse was lysed in 500 μl IPH lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1× complete EDTA-free protease inhibitor cocktail (Roche) using Kontes Pellet Pestle Motor and incubated on ice for 40 min. Cell debris was pelleted by centrifugation (17000 × g, 4 °C, 10 min) and the supernatants transferred to new eppendorf tubes. The protein concentration was determined by BCA Protein Assay Kit (Thermo Scientific) and the tissue lysates were subsequently diluted with IPH lysis buffer to an equal protein concentration of 25 μg/μl. The WT- and Naa10 KO tissue lysates were then divided in two, whereof one half was mixed with 15 μg of anti-Naa15 antibody and the other half with 15 μg of anti-V5 antibody as a negative control. The mixtures were incubated at 4 °C for 3 h on a rotator. Afterwards, 180 μl of Protein A/G magnetic beads (Millipore) pre-washed in IPH lysis buffer was added to each sample and incubated overnight. Then, the magnetic beads were washed three times in IPH lysis buffer and two times in 1x acetylation buffer (100 mM Tris-HCl pH 8.5, 2 mM EDTA, 20% Glycerol) prior to being resuspended in 90 μl of 2x Acetylation buffer and used in a [14C]-Ac-CoA–Based acetylation assay.

[14C]-Ac-CoA–based acetylation assay of immunoprecipitated samples. Three positive replicates were prepared for each IP sample containing 10 μl IP beads, 200 μM synthetic oligopeptide SESS24 (BioGenes), 100 μM [14C]-Ac-CoA (Perkin-Elmer) and dH2O to a final volume of 25 μl. In addition, two replicates for each IP sample were prepared without synthetic oligopeptide as negative controls. The samples were incubated at 37 °C for 45 min in a thermomixer with shaking at 1400 rpm. Finally, the magnetic beads were isolated and 23 μl of the supernatant transferred to P81 phosphocellulose filter discs (Millipore). The filter discs were washed three times for 5 min in 10 mM HEPES buffer (pH 7.4) and air dried. To determine the amount of incorporated [14C]-Ac, the filter discs were added to 5 mL Ultima Gold F scintillation mixture (Perkin-Elmer) and analysed by a Perkin-Elmer TriCarb 2900TR Liquid Scintillation Analyzer.

Proteomics sample preparation. Immunoprecipitation of Naa15 from a WT- and Naa10 KO mouse was performed as described above. Bound proteins were eluted from the magnetic beads using 60 μl of elution buffer (2% SDS, 100 mM Tris-HCl pH 7.6, 0.1 M DTT) and heated for 5 min at 95 °C. The eluates were processed for LC-MS/MS analysis using filter-aided sample preparation (FASP) method(86). The eluted protein mixtures were mixed with UA buffer (8 M urea, 100 Mm Tris-HCl pH 8.0) and centrifuged through Microcon 30kDa MWCO filters followed by Cys-alkylation with 50 mM iodoacetamide dissolved in UA buffer. Afterwards, the buffer was exchanged with 50 mM ammonium bicarbonate through sequential centrifugation, proteins were trypsinized (Sequencing Grade Modified Trypsin,
Promega) and digested peptides were collected by centrifugation. Peptides were acidified using 5% formic acid and desalted using C18-stagetips according to protocol(87). Briefly, 40 μg peptides from each sample were loaded onto C18-stagetips pre-conditioned with buffer A (1% formic acid). The C18-stagetips were then washed with buffer A, before peptides were eluted with buffer B (80% acetonitrile/ACN, 1% formic acid). The final eluate was concentrated by Speedvac to evaporate ACN and diluted to desired volume with 5% formic acid.

**Mass spectrometric analysis.** 1 μg of the peptide samples were injected into an Ultimate 3000 RSLC system (Thermo Scientific) connected to a Q-Exacte HF mass spectrometer (Thermo Scientific). Trapping and desalting was performed with 0.1% TFA (flow rate 5 μl/min, 5 min) on a pre-column (Acclaim PepMap 100, 2 cm x 75 μm ID nanoViper column, 3 μm C18 beads). Peptides were separated on an analytical column (PepMap RSLC, 50cm x 75 μm i.d. EASY-spray column, 2 μm C18 beads) during a biphasic ACN gradient with a flow rate of 200 nl/min. Solvent A (0.1% FA (vol/vol) in water) and B (100% ACN) were used for the following gradient composition: 5% B for 5 min, 5-8% B for 0.5 min, 8-24% B for 109.5 min, 24-35% B for 25 min and 35-80% B for 15 min, 80% B for 15 min and conditioning with 5% B for 20 min. The mass spectrometer was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. MS spectra (m/z 375-1500) were acquired with a resolution of 120 000 at m/z 200, automatic gain control (AGC) target of 3 x 106 and maximum injection time (IT) of 100 ms. The 12 most intense peptides above an intensity threshold (50 000 counts, charge states 2-5) were sequentially isolated to an AGC target of 1 x 105 and maximum IT of 100 ms and isolation width maintained at 1.6 m/z, before fragmentation at a normalised collision energy of 28%. Fragments were detected in the orbitrap at a resolution of 15 000 at m/z 200, with first mass fixed at m/z 100. Dynamic exclusion was utilized with an exclusion time of 2 s and “exclude isotopes” enabled. Lock-mass internal calibration (m/z 445.12003) was used. Raw files were processed with MaxQuant v. 1.5.5.1(88) and searched against a database of SwissProt annotated mouse protein sequences (retrieved 22.06.2018) in which the Naa12 sequence was added manually, and with a reverse decoy database. MaxQuant was run with default settings. Peptide and protein identifications were filtered to a 1% false discovery rate (FDR). Minimum peptide length was set to 7. Modifications included in protein quantification were oxidation (M), N-terminal acetylation, lysine acetylation, tryptophan hydroxylation and phosphorylation (STY). Other parameters: match between runs – true, matching time window – 0.7 min, alignment time window – 20 min, find dependent peptides – true, mass bin size – 0.0065.

**Whole body CT scanning.** CT scans were acquired on a Nanoscan PET/CT scanner from Mediso using Nuclene v2.01 software. All mice were kept sedated under isoflurane anesthesia for the duration of the scan. Scans were acquired with an X-ray tube energy and current of 70kVp and 280uA respectively. 720 projections were acquired per rotation, for 3 rotations, with a scan time of approximately 11 minutes, followed by reconstruction with a RamLak filter and voxel size 40x40x122μm. For ex vivo analyses, mouse heads were fixed in 10% formalin buffered saline, followed by scanning and reconstruction with 1440 projections per revolution. Cranial volume was measured using VivoQuant software (v2.50patch2), using the spline tool to manually and accurately draw around the circumference of the cranium on multiple stepwise 2D slices.

**Integrated N-terminal peptide enrichment (iNrich) assay.** iNrich assays were performed...
as described(43). Mouse embryonic fibroblasts (MEFs) were made from E13.5 embryos,
using standard techniques, with DMEM media supplemented with 10% fetal bovine serum
(FBS), L-glutamine and penicillin/streptomycin. Cells were harvested by trypsinization,
washed twice with ice-cold PBS (phosphate-buffered saline, pH 7.4; Gibco), and resuspended
in ice-cold lysis buffer containing 0.2 M EPPS (pH 8.0), 6 M guanidine, 10 mM TCEP
(Thermo Fisher Scientific), and 40 mM 2-chloroacetamide (Sigma-Aldrich). After 10 min of
incubation on 95 °C, cells were lysed by ultrasonication by a BranSonic 400B. The proteins
from the cell lysate were isolated by transferring supernatant after centrifugation at 12000g
for 10 min at 4 °C. The protein concentration of the collected supernatant was determined by
BCA (bicinchoninic acid) protein assay

**RNA and protein isolation and assays.** 70-120 mg tissues were lysed in 5 μl/mg tissue RIPA
buffer (Sigma) with 1x Complete protease inhibitors and 1 U/μl Superase In RNase inhibitor
(Thermo Scientific) using Fisherbrand Pellet Pestle Cordless Motor. After homogenization
debris was removed by centrifugation at 20.800 g for 10 min at 4°C. Protein concentration
was determined using APA assay (Cytoskeleton Inc.) and 50 μg total protein were separated
on SDS-PAGE followed by western blot. Membranes were stained with anti-Naa10, anti-
Naa15 and anti-GAPDH antibodies (all Abcam).

For RNA purification, 30 μl clarified lysate were mixed with 70 μl RNase free water and
RNA isolated using the RNeasy Mini Kit (Qiagen) according the manufacturers
recommendations, including on-column Dnase digest. 1 μg RNA was reverse transcribed
using the TaqMan Reverse transcription kit and gene level detection performed using SYBR
Green Master Mix (all Thermo Scientific). Relative expression was normalized to GAPDH
and ACTB.

For the characterization of the mNaa12 antibody, tissue was lysed in 2 μl per mg tissue PBS-
X (PBS + 0.2 % (v/v) Triton X-100 + 1 × Complete protease inhibitor cocktail). 10-200 μg
lysat were subjected to SDS-PAGE and western blot.

**Primers for mice qPCR.** The following primers pairs were used: mNaa10-Exon2/3-F: 5’-
ctcttgcccecgcttctt-3’ and mNaa10-Exon3/4-R: 5’- tegtctggtctctcttccat -3’, mNaa11-F: 5’-
accecaagaaagacagt-3’ and mNaa11-R: 5’- agcaagatgcaggaatgctct -3’,
mNaa12(Gm16286)-F: 5’-aegatgtgtaggaaga-3’ and mNaa12(Gm16286)-R: 5’-
cagaaagttgtcctctg-3’, mNaa15-F: 5’-gcagagcatggagaaaccct-3’ and mNaa15-R: 5’-
tcgtctgggtcctcttccat-3’, mNaa50-F: 5’-tagatgcctttgcaccttac-3’ and mNaa50-R: 5’-
gtaatactgtgactcttg-3’, mGAPDH-F: 5’-aggtaggggtggaagatt-3’ and mGAPDH-R: 5’-
tgtagacatgattgaggtca-3’, mACTB-F: 5’-ggttattcctccctcateg-3’ and mACTB-R: 5’-
ccagttggaatacgctgt-3’.

**Expression and purification of WT mouse, Naa10 and Naa12.** All constructs were
expressed in Rosetta (DE3)pLysS competent E. coli cells. Cells were grown in LB-media to
OD600 0.6-0.7 prior to inducing protein expression with 0.5 mM isopropyl β-D-1-
 thiogalactopyranoside (IPTG) at 18°C for ~16 hrs. All subsequent purification steps were
carried out at 4°C. Cells were isolated by centrifugation and lysed in lysis buffer containing
25 mM Tris, pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 10 μg/mL
phenylmethanesulfonylfluoride (PMSF), and DNase. The lysate was clarified by
centrifugation and incubated with amylase agarose resin (New England Biolabs) for 1 hour
before washing the resin with ≥100 column volumes of lysis buffer and then eluted with ten
column volumes of lysis buffer supplemented with 20 mM maltose. The resulting eluent was
pooled and concentrated to ~10 mg/mL (30 kDa concentrator; Amicon Ultra, Millipore) such that 500 µl was loaded onto a Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare). The gel filtration run was performed in sizing buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl, and 1 mM TCEP. Peak fractions running at ~14 mL were pooled, concentrated to 0.6 (6.1 µM) WT mouse Naa10 and 0.3 mg/mL (3.5 µM) WT mouse Naa12, as measured by UV₂₈₀ (Nanodrop 2000; Thermo Fisher Scientific), and stored at 4°C.

In vitro radioactive acetyltransferase assays. The assays were carried out in 40 mM HEPES, pH 7.5 200 mM NaCl, where reactions were incubated with 150 nM of the gel-filtration purified WT mouse Naa10 or Naa12 in a 30 µl reaction volume containing each 250 µM substrate peptide and radiolabeled [¹⁴C]acetyl-CoA (4 mCi/mmol; PerkinElmer Life Sciences) for 12 min (Naa12) or 13 min (Naa10) at 25°C. Respective time points were selected to ensure detection of sufficient activity within the linear range as determined by a time course experiment. The substrate peptides used in the assay corresponds to the first 7 amino acids of β-actin (DDDAAL-), γ-actin (EEEIAAL-), or the in vitro NtaA complex substrate High mobility group protein A1(SESSS-) , along with C-terminal positively charged residues for capture to the anion exchange paper. Background control reactions were performed in the absence of enzyme or in the absence of substrate peptide to ensure that any possible signal due to chemical acetylation was negligible. Each reaction was performed in triplicate.

To quench the reaction, 20 µl of the reaction mixture was added to negatively charged P81 phosphocellulose squares (EMD Millipore), and the paper disks were immediately placed in wash buffer (10 mM HEPES, pH 7.5). The paper disks were washed three times, at 5 min per wash, to remove unreacted acetyl-CoA. The papers were then dried with acetone and added to 4 mL of scintillation fluid, and the signal was measured with a PerkinElmer Life Sciences Tri-Carb 2810 TR liquid scintillation analyzer. The counts per minute were converted to molar units using a standard curve of known [¹⁴C]acetyl-CoA concentrations in scintillation fluid.

Full peptide sequences:

β-actin: NH2-DDDAALRWGPRVGRPRPVRVYP-COOH
γ-actin: NH2-EEEIAALRWGPRVGRPRPVRVYP-COOH
Histone H4: NH2-SGRGKGGKGLGKGGAKRHR-COOH

Statistical analyses. Significant differences (p<0.05) are indicated by asterisks. Weight analyses were performed using generalized estimating equations (GEEs) (89), an extension of generalized linear models which adjusts for the effects of autocorrelation resulting from multiple measurements, and implemented within version 15.1 of Stata (Statacorp 2017).

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Competing interests
The authors declare no competing interest.

References


50. Park EC, Szostak JW. ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. EMBO J. 1992;11(6):2087-93.


Supporting information

S1 Table. Genotypes of offspring from Naa10<sup>+/−</sup> female mice crossed to the Naa10<sup>+/Y</sup> male mice.

<table>
<thead>
<tr>
<th>Genotype (Expected Mendelian %)</th>
<th>Naa10&lt;sup&gt;+/Y&lt;/sup&gt; or Naa10&lt;sup&gt;++&lt;/sup&gt; (50%)</th>
<th>Naa10&lt;sup&gt;+/−&lt;/sup&gt; (25%)</th>
<th>Naa10&lt;sup&gt;−/−&lt;/sup&gt; (25%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5 (n=134)</td>
<td>62 (46.3%)</td>
<td>39 (29.1%)</td>
<td>33 (24.6%)</td>
</tr>
<tr>
<td>E13.5 (n=98)</td>
<td>53 (54.1%)</td>
<td>22 (22.4%)</td>
<td>23 (23.4%)</td>
</tr>
<tr>
<td>E18.5 (n=170)</td>
<td>82 (48.2%)</td>
<td>49 (28.8%)</td>
<td>39 (23.0%)</td>
</tr>
<tr>
<td>Adults (n=733)</td>
<td>438 (59.8%)</td>
<td>207 (28.2%)</td>
<td>88 (12.0%)</td>
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</tbody>
</table>

Expected and observed Mendelian ratio of genotypes in offspring at E10.5, E13.5, E18.5 and adults from crosses of Naa10<sup>+/−</sup> female and Naa10<sup>+/Y</sup> male mice. The percentage of adult Naa10<sup>−/Y</sup> mice significantly decreases.

S2 Table. Genotypes of offspring from Naa10<sup>+/tm1a</sup> female mice crossed to the Naa10<sup>+/Y</sup> male mice.

<table>
<thead>
<tr>
<th>Genotype (Expected Mendelian %)</th>
<th>Naa10&lt;sup&gt;+/Y&lt;/sup&gt; or Naa10&lt;sup&gt;++&lt;/sup&gt; (50%)</th>
<th>Naa10&lt;sup&gt;+/tm1a&lt;/sup&gt; (25%)</th>
<th>Naa10&lt;sup&gt;tm1a/Y&lt;/sup&gt; (25%)</th>
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<tr>
<td>E10.5 (n=109)</td>
<td>55 (50.46%)</td>
<td>26 (23.85%)</td>
<td>28 (25.69%)</td>
</tr>
<tr>
<td>E12.5 (n=45)</td>
<td>20 (44.4%)</td>
<td>12 (26.7%)</td>
<td>13 (28.8%)</td>
</tr>
<tr>
<td>E18.5 (n=53)</td>
<td>27 (51.0%)</td>
<td>13 (24.5%)</td>
<td>13 (24.5%)</td>
</tr>
<tr>
<td>Adults (n=260)</td>
<td>152 (58.5%)</td>
<td>85 (32.7%)</td>
<td>23 (08.8%)</td>
</tr>
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</table>

Expected and observed Mendelian ratio of genotypes in offspring at E10.5, E13.5, E18.5 and adults from crosses of Naa10<sup>+/tm1a</sup> female and Naa10<sup>+/Y</sup> male mice. The percentage of adults Naa10<sup>tm1a/Y</sup> mice significantly decreases.

S3 Table. Cervical fusion skeletal analyses in Naa10 KO mice.

<table>
<thead>
<tr>
<th>genotype</th>
<th>sample size</th>
<th>one or more fusion events</th>
<th>two or more fusion events</th>
<th>consecutive fusion events</th>
<th>C1+2 fusion</th>
<th>C2+3 fusion</th>
<th>C3+4 fusion</th>
<th>C4+5 fusion</th>
<th>C5+6 fusion</th>
<th>C6+7 fusion</th>
<th>C7+ T1 fusion</th>
<th>T1+2 fusion</th>
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<tbody>
<tr>
<td>Naa10&lt;sup&gt;+/Y&lt;/sup&gt;</td>
<td>19</td>
<td>2/17 (12%)</td>
<td>1/17 (6%)</td>
<td>0/17 (0%)</td>
<td>2/17 (12%)</td>
<td>0/18 (0%)</td>
<td>0/18 (0%)</td>
<td>0/19 (0%)</td>
<td>0/19 (0%)</td>
<td>1/19 (5%)</td>
<td>0/19 (0%)</td>
<td></td>
</tr>
<tr>
<td>Naa10&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>4</td>
<td>1/4 (25%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>1/4 (25%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td></td>
</tr>
<tr>
<td>Naa10&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4</td>
<td>1/4 (25%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>1/4 (25%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td></td>
</tr>
<tr>
<td>Naa10&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
<td>9/10 (90%)</td>
<td>3/9 (33%)</td>
<td>1/9 (11%)</td>
<td>7/10 (70%)</td>
<td>2/9 (22%)</td>
<td>2/9 (22%)</td>
<td>1/9 (11%)</td>
<td>0/9 (0%)</td>
<td>1/9 (11%)</td>
<td>0/9 (0%)</td>
<td></td>
</tr>
<tr>
<td>Naa10&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
<td>0/1 (0%)</td>
<td>0/1 (0%)</td>
<td>0/1 (0%)</td>
<td>0/1 (0%)</td>
<td>0/1 (0%)</td>
<td>0/1 (0%)</td>
<td></td>
</tr>
</tbody>
</table>
S4 Table. Matings and litter size analyses.

<table>
<thead>
<tr>
<th>Genotypes of breeders (♀ x ♂)</th>
<th>Naa10&lt;sup&gt;+/+&lt;/sup&gt; x Naa10&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;+/+&lt;/sup&gt; x Naa10&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;-/+&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Naa10&lt;sup&gt;-/-&lt;/sup&gt; x Naa10&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>#pups</td>
<td>255</td>
<td>255</td>
<td>330</td>
<td>59</td>
<td>59</td>
<td>127</td>
<td>191</td>
<td>21</td>
</tr>
<tr>
<td>#litters</td>
<td>39</td>
<td>18</td>
<td>33</td>
<td>59</td>
<td>59</td>
<td>127</td>
<td>191</td>
<td>51</td>
</tr>
<tr>
<td>#pups/#litters, or litter size</td>
<td>6.5</td>
<td>9.0</td>
<td>5.0</td>
<td>4.5</td>
<td>5.4</td>
<td>4.1</td>
<td>5.0</td>
<td>4.2</td>
</tr>
<tr>
<td>SD of litter size</td>
<td>3.2</td>
<td>0.0</td>
<td>2.2</td>
<td>2.5</td>
<td>2.5</td>
<td>2.1</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>% Died in 1st three days of life</td>
<td>5.1%</td>
<td>5.6%</td>
<td>15.8%</td>
<td>13.6%</td>
<td>42.4%</td>
<td>36.0%</td>
<td>18.8%</td>
<td>28.6%</td>
</tr>
<tr>
<td>% of total that died by weaning ~4 weeks</td>
<td>5.9%</td>
<td>11.1%</td>
<td>23.0%</td>
<td>32.2%</td>
<td>59.3%</td>
<td>44.0%</td>
<td>22.0%</td>
<td>33.3%</td>
</tr>
<tr>
<td>Avg Length of Mating till 1st Litter:</td>
<td>29</td>
<td>22</td>
<td>34</td>
<td>25</td>
<td>35</td>
<td>28</td>
<td>26</td>
<td>63</td>
</tr>
<tr>
<td>Total number of unique mating males:</td>
<td>7</td>
<td>1</td>
<td>12</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Total number of mating pairs set up:</td>
<td>8</td>
<td>1</td>
<td>&gt;16</td>
<td>14</td>
<td>10</td>
<td>11</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>Total number of mating pairs with progeny</td>
<td>7</td>
<td>1</td>
<td>N/A</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>% females who became pregnant and gave birth at least once:</td>
<td>87.5%</td>
<td>100.0%</td>
<td>N/A</td>
<td>50.0%</td>
<td>40.0%</td>
<td>100.0%</td>
<td>95.5%</td>
<td>71.4%</td>
</tr>
</tbody>
</table>

Naa10 KO matings, all WT/WT for Naa12, all >99.6% C57BL/6J, mixed genetic background.
### S5 Table. Genotypes of offspring from Naa12\(^{+/-}\) female mice crossed to the Naa12\(^{+/-}\) male mice.

<table>
<thead>
<tr>
<th>Genotype (Expected Mendelian %)</th>
<th>Naa12(^{+/-}) (25%)</th>
<th>Naa12(^{-/-}) (50%)</th>
<th>Naa12(^{-/-}) (25%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (n=117)</td>
<td>26 (22%)</td>
<td>62 (53%)</td>
<td>29 (25%)</td>
</tr>
</tbody>
</table>

Expected and observed Mendelian ratio of genotypes in offspring from crosses of Naa12\(^{+/-}\) female and Naa12\(^{+/-}\) male mice.

### S6 Table. Mice analyzed by weighing, according to genotype.

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naa12 status</td>
<td>Naa12 status</td>
</tr>
<tr>
<td></td>
<td>WT/WT</td>
<td>WT/KO</td>
</tr>
<tr>
<td>Naa10 status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure C57BL/6J</td>
<td>WT/WT</td>
<td>67</td>
</tr>
<tr>
<td>background Naa10</td>
<td>WT/KO</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>KO/KO</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>207</td>
</tr>
<tr>
<td>mixed genetic</td>
<td>WT/WT</td>
<td>32</td>
</tr>
<tr>
<td>background Naa10</td>
<td>WT/KO</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>KO/KO</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>274</td>
</tr>
</tbody>
</table>

Mixed genetic background Naa10 and Naa12 mice

Total: 389 males, 299 females

35
### S7 Table. Effects of Naa10 KO on growth rate of Naa10 mice on pure genetic background.

#### C57BL/6J inbred females (N = 207)

<table>
<thead>
<tr>
<th>Effect of age and age$^2$</th>
<th>Effect of Naa10 KO</th>
<th>Effect of age and Naa10 KO</th>
<th>Effect of age, Naa10 KO, and interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coeff. SE z p &gt;</td>
<td>z</td>
<td>Coeff. SE z p &gt;</td>
<td>z</td>
</tr>
<tr>
<td>Age in days</td>
<td>0.349 0.006 59.49 &lt; 0.001</td>
<td>0.349 0.006 59.38 &lt; 0.001</td>
<td>0.344 0.010 34.59 &lt; 0.001</td>
</tr>
<tr>
<td>Age$^2$</td>
<td>-0.001 0.00003 -40.59 &lt; 0.001</td>
<td>-0.001 0.00003 -40.56 &lt; 0.001</td>
<td>-0.001 0.00005 -24.53 &lt; 0.001</td>
</tr>
<tr>
<td>Naa10 KO</td>
<td>-2.92 0.847 -3.45 0.001</td>
<td>-2.52 0.248 -1.01 ns</td>
<td>-0.219 0.451 -0.49 ns</td>
</tr>
<tr>
<td>Age x KO</td>
<td>17.23 0.690 24.96 &lt; 0.001</td>
<td>2.697 0.272 9.93 &lt; 0.001</td>
<td>2.633 0.382 6.90 &lt; 0.001</td>
</tr>
<tr>
<td>(constant)</td>
<td>2.518 0.204 12.35 &lt; 0.001</td>
<td>2.518 0.204 12.35 &lt; 0.001</td>
<td>2.518 0.204 12.35 &lt; 0.001</td>
</tr>
</tbody>
</table>

Wald $\chi^2$ a | 6547.29, p < 0.0001 | 6552.24, p < 0.0001 | 6611.63, p < 0.0001 |

---

#### C57BL/6J inbred males (N = 167)

<table>
<thead>
<tr>
<th>Effect of age and age$^2$</th>
<th>Effect of Naa10 KO</th>
<th>Effect of age and Naa10 KO</th>
<th>Effect of age, Naa10 KO, and interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coeff. SE z p &gt;</td>
<td>z</td>
<td>Coeff. SE z p &gt;</td>
<td>z</td>
</tr>
<tr>
<td>Age in days</td>
<td>0.454 0.008 60.16 &lt; 0.001</td>
<td>0.454 0.007 62.20 &lt; 0.001</td>
<td>0.467 0.009 51.68 &lt; 0.001</td>
</tr>
<tr>
<td>Age$^2$</td>
<td>-0.002 0.00005 -39.76 &lt; 0.001</td>
<td>-0.002 0.00004 -41.16 &lt; 0.001</td>
<td>-0.002 0.00005 -34.53 &lt; 0.001</td>
</tr>
<tr>
<td>Naa10 KO</td>
<td>-4.721 1.040 -4.54 &lt; 0.001</td>
<td>-2.578 0.304 -8.47 &lt; 0.001</td>
<td>-1.351 0.504 -2.68 0.007</td>
</tr>
<tr>
<td>Age x KO</td>
<td>19.56 0.668 29.28 &lt; 0.001</td>
<td>2.430 0.283 8.58 &lt; 0.001</td>
<td>1.931 0.321 6.01 &lt; 0.001</td>
</tr>
<tr>
<td>(constant)</td>
<td>1.303 0.271 4.81 &lt; 0.001</td>
<td>1.303 0.271 4.81 &lt; 0.001</td>
<td>1.303 0.271 4.81 &lt; 0.001</td>
</tr>
</tbody>
</table>

Wald $\chi^2$ a | 7220.56, p < 0.0001 | 8007.09, p < 0.0001 | 8185.75, p < 0.0001 |

---

[a] The Wald $\chi^2$ is a measure of the overall goodness of fit of the complete model.
### S8 Table. Effects of Naa10 and Naa12 KOs on growth rate on mixed genetic background

<table>
<thead>
<tr>
<th>Effect: age and Naa10 KO</th>
<th>Effect: age and Naa12 KO</th>
<th>Effect: age, Naa10 &amp; Naa12 KOs</th>
<th>Effect: age, Naa10, Naa12, both KOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coeff</td>
<td>SE</td>
<td>z</td>
<td>p &gt;</td>
</tr>
<tr>
<td>Age</td>
<td>0.058</td>
<td>0.013</td>
<td>29.24</td>
</tr>
<tr>
<td>Age²</td>
<td>-0.003</td>
<td>0.0001</td>
<td>-22.76</td>
</tr>
<tr>
<td>Naa10</td>
<td>0.109</td>
<td>0.047</td>
<td>2.20</td>
</tr>
<tr>
<td>Age x Naa10</td>
<td>-0.059</td>
<td>0.023</td>
<td>-2.51</td>
</tr>
<tr>
<td>Age² x Naa10</td>
<td>0.0006</td>
<td>0.0002</td>
<td>2.25</td>
</tr>
<tr>
<td>Naa12-Het</td>
<td>-0.175</td>
<td>0.547</td>
<td>-0.32</td>
</tr>
<tr>
<td>Age x Naa12-Het</td>
<td>-0.005</td>
<td>0.023</td>
<td>-0.23</td>
</tr>
<tr>
<td>Age² x Naa12-Het</td>
<td>0.00004</td>
<td>0.0002</td>
<td>0.02</td>
</tr>
<tr>
<td>Naa12-Ho</td>
<td>-0.882</td>
<td>1.584</td>
<td>-0.56</td>
</tr>
<tr>
<td>Age x Naa12-Ho</td>
<td>0.003</td>
<td>0.073</td>
<td>0.04</td>
</tr>
<tr>
<td>Age² x Naa12-Ho</td>
<td>0.0003</td>
<td>0.0007</td>
<td>0.04</td>
</tr>
<tr>
<td>Naa10-Naa12-Het</td>
<td>0.784</td>
<td>1.303</td>
<td>0.60</td>
</tr>
<tr>
<td>Age x Naa10-Naa12-Het</td>
<td>-0.113</td>
<td>0.052</td>
<td>-2.15</td>
</tr>
<tr>
<td>Age³ x Naa10-Naa12-Het</td>
<td>0.0009</td>
<td>0.0005</td>
<td>1.73</td>
</tr>
<tr>
<td>(constant)</td>
<td>0.588</td>
<td>0.305</td>
<td>1.93</td>
</tr>
</tbody>
</table>

| Wald X² | df | p > |z| |
|---------|----|---------|
| Naa10-Naa12-Het | 6224.04 | p < 0.0001 |
| Naa10-Naa12-Het | 4752.14 | p < 0.0001 |
| Naa10-Naa12-Het | 5528.71 | p < 0.0001 |
| Naa10-Naa12-Het | 5586.86 | p < 0.0001 |

Het : heterozygous
Ho : homozygous

*a The Wald X² is a measure of the overall goodness of fit of the complete model.
**S1 Fig. Generation and confirmation of Naa10tm1a mice.** (A) Schematic illustration of the Naa10tm1a mice. (B) PCR confirmation of Naa10 deficiency. (C) Confirmation of Naa10 protein in kidney tissue by Western blot. Naa10 protein is not detected in Naa10-/- mouse. (D) Expression pattern of Naa10 in the embryo. β-gal staining represent Naa10 localization.
**S2 Fig. Gross anatomy and histology of neonatal mouse hearts.** (A) Wildtype male heart outflow tract region indicating separate aorta and pulmonary trunks nestled between left and right atria. (B) Naa10−/− female heart from dying P0 pup only has a single outflow tract emerging from the right ventricle, resulting in persistent truncus arteriosus. (C) Naa10−/− male heart from dying P0 pup has separate outflow tracts but both emerge from the right ventricle, resulting in double outlet right ventricle. (D) normal histology from wildtype male heart of pulmonary artery exiting the right ventricle. (E) histology from Naa10−/− heart of single outflow tract exiting the right ventricle with tricuspid valve leaflets. (F) histology from Naa10−/− heart of both pulmonary and aortic arteries emerging from right ventricle within the same plane. (G) Naa10−/− histology showing membranous VSD, (H) Naa10−/− histology showing muscular VSD. (I) normal heart revealing closed ductus arteriosus, J) Naa10−/− histology showing open ductus arteriosus leading to pulmonary overload and likely lethality.
S3 Fig. Skeletal phenotype by CT scanning. (A) In WT mice 13 thoracic vertebrae and ribs are numbered whereas 14 thoracic vertebrae and ribs are counted in mutants (Naa10<sup>−/−</sup>) (WT on the left, mutant on the right). n=11 CT scans for Naa10<sup>−/−</sup> compared to n=18 Naa10<sup>+/−</sup>. (B-D) Different number of ribs are linking the sternum between in Naa10<sup>−/−</sup>, Naa10<sup>−/−</sup> and WT. (B) 7 ribs linking the sternum in WT. (C) 8 ribs linking the sternum (the white arrow shows the 8th rib) in Naa10<sup>−/−</sup>. (D) 7 on one side + 7 and one almost linking on the other side. In 2 mice, an asymmetrical link was observed. White arrow shows the eighth asymmetrical rib. (E and F) Abnormalities in the cervical phenotype. (E) Cervical WT/ morphology. (F) Partial fusion of C1 and C2 dorsal arch in one mutant mouse.
S4 **Fig. Hydronephrosis and Hydrocephaly in Naa10 KO mice.** (A) Representative images and histology of renal defects at E18.5 (n=6 out of 39 examined) and P3 (n=4 out of 11 examined). (B) Hydrocephaly (n=3 CT scans for Naa10−/− mice with hydrocephaly compared to n=3 Naa10−/− mice without hydrocephaly).
S5 Fig. Identification of a potential Naa10 homolog. (A) Construction of Naa10 Southern blot probe. (B) Southern blot membrane after hybridization with a Naa10 probe. Expected size band, restricted with Apa I and Xba I, were showed. (C) Ribosome profiling traces for the potential Naa10 paralog (Gm16286, UniProt: Q9CQX6). Picture was modified from GWIPS genome browser, Chr 18, 80206601-80212942.
S6 Fig. Characterization of a potential Naa10 homolog. (A) qPCR analyses of mouse NATs (mNATs) in Naa10 WT and KO mouse tissue. (B) Sequence alignment of mNaa10 isoforms and paralogs, including the potential Naa10 paralog mNaa12 (Gm16286, UniProt: Q9CQX6) using Clustal Omega (EMBL-EBI). The peptide used for immunization of rabbits to generate a specific antibody is indicated in red. (C) Full length mNAT cDNA from mouse was amplified and cloned into pGEX-4T1. Proteins were expressed in E. coli BL21 (DE3) and purified via GSH-sepharose. Shown is a coomassie stain of fraction 1-4. (D) Cross-reactivity and sensitivity of the used NAT antibodies. 1-20 pmol of GST-mNAT proteins were separated on SDS-PAGE followed by western blot, probed with the indicated antibodies. (E) Detection of endogenous mNaa12. Tissue lysate from liver, kidney and heart were subjected to SDS-PAGE and WB. Right panel of Naa12 shows antibody competition assay.
**S7 Fig. Confirmation and characterization of Naa12 KO mice.** (A) Expression of Naa10 and Naa12 in WT, Naa10 KO and Naa12 KO tissues (brain, heart, kidney and testis) by RT-PCR. Expression of GAPDH was analyzed as an endogenous control. (B) Expression of Naa12 protein determined in WT, Naa10 KO and Naa12 KO tissues (brain, heart, kidney, lung, liver and testis) by Western blot analysis. (C) Phenotypes in Naa12 KO mice. Lack of hypopigmentation (upper; N=29) and lack of supernumerary ribs (middle and bottom; E18.5; N=7) in Naa12 KO mouse.
**S8 Fig. Genotypes of offspring from** *Naa10*+/−; *Naa12*+/− **female mice crossed to the** *Naa10*+/Y; *Naa12*−/− **male mice.** *Naa10; Naa12* DKO exhibit embryonic lethality. Pedigree of mating and genotypes of pups and embryos at E8.5, E10.5, E12.5 and E18.5.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5 (n=21)</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>E10.5 (n=28)</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>E12.5 (n=25)</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>E18.5 (n=11)</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Postnatal (n=179)</td>
<td>40</td>
<td>45</td>
<td>47</td>
<td>35</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* lyses embryo