Indeed, we were able to confirm that overexpressed Nmnat3 was localized in mitochondria in HeLa cells (Fig. 1B). However, it is also known that mature erythrocytes have no mitochondria. We accordingly investigated the subcellular localization of Nmnat3 in mature erythrocytes. We isolated mature erythrocytes from the wild-type mice by a Percoll gradient method and then successfully fractionated mature erythrocyte proteins into cytoplasmic and membrane fractions. We found Nmnat3 exclusively in the cytoplasmic fraction of mature erythrocyte (Fig. 1C). Next, we examined the subcellular localization of Nmnat3 in reticulocyte, which possess mitochondria. We found that Nmnat3 in reticulocytes also existed in cytoplasmic fraction rather than mitochondria (Fig. 1D). From these results, we concluded that Nmnat3 is a cytoplasmic protein, at least in mature erythrocytes and reticulocytes.

Nmnat3$^{gt/gt}$ mice exhibited splenomegaly and hemolytic anemia

To investigate the physiological functions of Nmnat3, we generated Nmnat3-deficient mice by the gene-trap method (Fig. 2A-C). We verified that Nmnat3 mRNA and protein were absent in Nmnat3 gene-trap homozygous (Nmnat3$^{gt/gt}$) mice (Fig. 2D-H). Thus, Nmnat3$^{gt/gt}$ mice were considered as Nmnat3-deficient mice, or at least severely Nmnat3-scarce mice.

Nmnat3$^{gt/gt}$ mice were born in a Mendelian's ratio (data not shown) and looked normal at appearance. However, Nmnat3$^{gt/gt}$ mice exhibited splenomegaly from a young age (Fig. 2I and J), and histological examination revealed that the red pulp of the spleen was enlarged (Fig. 2K). Increased iron deposits (hemosiderin) were also seen in Nmnat3$^{gt/gt}$ mice spleens (Fig. 2K). These results suggested that erythrocyte destruction was increased in Nmnat3$^{gt/gt}$ mice. As expected, hematological examination of peripheral blood in Nmnat3$^{gt/gt}$ mice revealed moderate anemia, as evidenced by decreased numbers of RBC, lower hematocrits (Ht) and lower concentrations of hemoglobin (Hb) (Table 2). However, no significant differences were found in numbers of white blood cell (WBC) and platelets (PLT) between Nmnat3$^{gt/gt}$ and control mice. Numbers of reticulocytes in Nmnat3$^{gt/gt}$ mice were markedly increased (Table 2). We confirmed reticulocytosis in Nmnat3$^{gt/gt}$ mice by May–Giemsa staining and New Methylene Blue staining (Fig. 2L). These data suggested that Nmnat3$^{gt/gt}$ mice manifested hemolytic anemia. In support of this view, scanning electron microscopy (SEM) detected a striking morphological change in Nmnat3$^{gt/gt}$ RBCs. RBCs in control mice showed the normal discocyte shape, whereas most RBCs in Nmnat3$^{gt/gt}$ mice appeared as acanthocytes or echinocytes, which show abnormal spiked membrane projections (Fig. 2M). Next, we assessed the lifespan of erythrocytes using an *in vivo* biotin-labeling method. The lifespan of mature erythrocytes in rodents has been reported as 50 to 60 days on an average (37). However, we found the lifespan of erythrocytes in Nmnat3$^{gt/gt}$ mice to be greatly reduced to approximately 10 days (Fig. 2N). We concluded from these findings that Nmnat3$^{gt/gt}$ mice exhibited splenomegaly due to hemolytic anemia.

Erythropoiesis is upregulated in Nmnat3$^{gt/gt}$ mice as a compensatory mechanism

Nmnat3$^{gt/gt}$ mice showed increased numbers of reticulocytes (Table 2 and Fig. 2L), suggesting that erythropoiesis in these mice would be normal. In anemic mice, increased erythropoiesis primarily occurs in spleen rather than in bone marrow (45). We next investigated the erythropoiesis stage using the cell surface markers CD71 (transferrin receptor) and Ter119 (glycophorin-associating protein) in splenocytes and bone marrow cells (46). Immature CD71^{high}Ter119^{high} (P4) and CD71^{middle}Ter119^{high} (P5) fractions were markedly increased in splenocytes from Nmnat3$^{gt/gt}$ mice (Fig. 3A). However, the most mature CD71^{low}Ter119^{high} (P6) fraction was decreased. The larger P4 fraction was also observed in bone marrow cells (Fig. 3B). We further checked the erythroid developmental stage of peripheral blood. Consistent with the hematological examination, mature erythrocyte fraction (P6) was decreased in Nmnat3$^{gt/gt}$ mice (Fig. 3C). In general, reticulocytes enter the peripheral blood after enucleation and retain mitochondria. We investigated whether this step occurred normally in Nmnat3$^{gt/gt}$ mice. Peripheral blood samples from Nmnat3$^{gt/gt}$ and control mice were stained

with Hoechst 33342 and MitoTracker Orange for nucleus and mitochondria, respectively. As shown in Fig. 3D, nuclei were rarely seen in either mice, but there were more mitochondria in Nmnat3$^{gt/gt}$ mice likely due to larger fraction of reticulocytes. Taking all evidence together, we concluded that erythropoiesis was markedly increased to compensate for the decrease in mature erythrocytes, but that peripheral erythroid maturation was generally normal in Nmnat3$^{gt/gt}$ mice. These results also support the proposition that the anemia in Nmnat3$^{gt/gt}$ mice was not due to the abnormality in erythropoiesis but due to the shorter lifespan of mature erythrocytes.

Nmnat3 is a dominant Nmnat isozyme in mature erythrocytes

We found that Nmnat3 was present in mature erythrocytes and had a crucial role in their lifespan regulation. However, the significance of the two other Nmnat isozymes in mature erythrocytes is not clear. To address this question, we investigated whether enzymatic activity of the other Nmnat isoforms could be detected in mature erythrocytes. To assess Nmnat enzymatic activity, we employed an Nmnat isozyme discrimination assay (38), in which $MgCl_2$ was used as a metal cofactor to measure total Nmnat activity and $CoCl_2$ for relatively Nmnat3-specific activity. We confirmed that Nmnat3-specific activity was almost absent from Nmnat3$^{gt/gt}$ erythrocytes (Fig. 4A, Left panel). Total Nmnat activity was largely compromises as well, suggesting that the other two Nmnat activities were absent or at least very minor (Fig. 4A, Right panel). Consistent with these results, neither Nmnat1 nor Nmnat2 was identified in mature erythrocytes by Western blotting with Nmnat1- and Nmnat2-specific antibodies (Fig. 4B). These results suggested that Nmnat3 is most dominant among the three Nmnat isozymes in mature erythrocytes.

NAD pool is drastically decreased in Nmnat3$^{gt/gt}$ erythrocytes

Next, we investigated the effect of Nmnat3 deficiency on NAD metabolism in erythrocytes. We measured NAD-related metabolites in RBCs using multiple reaction monitoring (MRM)-based liquid chromatography–tandem mass spectrometry (LC-MS/MS) method. The NAD level in Nmnat3$^{gt/gt}$ erythrocytes was drastically reduced compared with that in wild-type erythrocytes (Fig. 5A and B). NADH was also markedly decreased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 5C). However, NADH/NAD ratio was not much changed (WT 0.0072; Nmnat3$^{gt/gt}$ 0.0063). To avoid the potential changes during sample preparation, we also measured NAD in whole blood samples, which were immediately quenched after blood collection from anesthetized mice. NAD levels of whole blood in Nmnat3$^{gt/gt}$ mice were reduced to the same extent as in RBCs (Fig. 5D). We also measured other NAD-related metabolites in RBCs and whole blood. NMN and NAM were markedly reduced in Nmnat3$^{gt/gt}$ erythrocytes. (Fig. 5E and F). However, nicotinic acid (NA) and tryptophan (Trp) were unchanged between WT and Nmnat3$^{gt/gt}$ RBCs. We also found that nicotinic acid dinucleotide (NAAD) was significantly reduced in Nmnat3$^{gt/gt}$ RBCs (Fig. 5E). This result confirmed the involvement of Nmnat3 in the final step of the *de novo* synthesis pathway. In addition, we measured NAD level in other tissues, such as liver, heart and skeletal muscle and surprisingly found that the level of NAD in these tissues was not changed in Nmnat3$^{gt/gt}$ mice (Fig. 5G). We also determined whether the NAD level in erythroid precursor cells was affected. We isolated P4 (polychromatophilic erythroblast, orthochromatic erythroblast and reticulocyte), P5 (reticulocyte) and P6 (mature erythrocyte) subpopulation cells from total splenocytes using a cell sorter (Fig. 5H). Consistent with the result obtained from RBCs and whole blood, the mature erythrocytes fraction (P6) from Nmnat3$^{gt/gt}$ mice had a markedly reduced NAD level. In contrast, no change was observed in the P4 or P5 fractions (Fig. 5I). These results suggested that Nmnat3 is critical for maintaining NAD levels only in mature erythrocytes and not in their precursor cells or other tissues such as heart, liver and skeletal muscle.

In erythrocytes, the pentose phosphate pathway is very active in protecting against oxidative stress through the NADP–glutathione system (47). Interestingly, we observed no significant difference in NADP level between WT and Nmnat3$^{gt/gt}$ erythrocytes (Fig. 5J). To investigate

how NADP was maintained in Nmnat3$^{gt/gt}$ erythrocytes, we examined the expression level of NAD kinase (NADK), which was responsible for generating NADP from NAD. We found that NADK was significantly increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 5K). In addition, the concentration of NADP in erythrocyte was much less than that of NAD (Fig. 5B and J). Therefore, it was speculated that NADP in Nmnat3$^{gt/gt}$ erythrocytes was more preferentially maintained than NAD. We also found that reduced glutathione (GSH) level in Nmnat3$^{gt/gt}$ erythrocytes was comparable to that in WT. However, the oxidative glutathione (GSSG) was slightly but significantly decreased (Fig. 5L). This result also supported the idea that NADP was maintained in Nmnat3$^{gt/gt}$ erythrocytes. Taken all together, we inferred that the NADP–glutathione system in Nmnat3$^{gt/gt}$ erythrocytes was not impaired.

Glycolytic pathway was blocked in Nmnat3$^{gt/gt}$ erythrocytes

Erythrocytes are known to exclude all of organelles, including nucleus and mitochondria, during maturation. Thus, energy (as ATP) production in mature erythrocytes relies primarily on the cytosolic glycolysis pathway. In particular, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) use NAD and NADH for their enzymatic reactions and recycle them between these two reactions. To assess the effect of Nmnat3 deficiency on glycolysis, we quantified metabolites of the glycolysis pathway and the pentose phosphate pathway by LC-MS/MS. Among glycolysis pathway intermediate metabolites, upstream metabolites, including glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and fructose 1,6-bisphosphate (F1,6BP) were markedly increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6A). In contrast, downstream metabolites, including 3-phosphoglycerate (3PG), phosphoelolpyruvate (PEP), and lactate (LAC) were significantly decreased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6A). In addition, phosphoribosyl pyrophosphate (PRPP) was markedly increased (Fig. 6A). In the pentose phosphate pathway, ribose 5-phosphate (R5P), ribulose 5-phosphate (Ru5P), and xylulose 5-phosphate (Xu5P) were increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6A). These data indicated that the glycolysis pathway in Nmnat3$^{gt/gt}$ erythrocytes was blocked in the middle and that the metabolic flow was shifted to the pentose phosphate pathway.

Next, we examined enzymatic activities of various glycolytic enzymes in erythrocytes to determine how the glycolysis pathway in Nmnat3$^{gt/gt}$ erythrocytes was blocked. We measured enzymatic activities of RBC lysate with excess amounts of substrates *in vitro*. The activities of most enzymes were upregulated in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6B). For instance, phosphofructokinase (PFK), a rate-limiting enzyme of glycolysis, was markedly increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6B). In agreement with this finding, the protein level of PFK was also increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6C). We also investigated other enzymes whose deficiency causes hemolytic anemia in human patients. However, they were unchanged or even increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6D and E). Previous study reported that acetylation increased the enzymatic activities of certain glycolytic enzymes (4,5). In Nmnat3$^{gt/gt}$ erythrocytes, NAD levels were markedly decreased, and NAD-dependent deacetylase sirtuins may have been inactivated. Additionally, levels of acetyl-CoA, which is a substrate for acetylation, were also markedly increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6F). We accordingly speculated that the acetylation status of Nmnat3$^{gt/gt}$ erythrocyte proteins would be increased. We examined acetylation status by Western blotting with antibodies against acetylated-lysine residues and found that acetylation levels were indeed increased in Nmnat3$^{gt/gt}$ erythrocytes compared with that in WT (Fig. 6G). We inferred that the increased activities of several glycolytic enzymes in Nmnat3$^{gt/gt}$ erythrocytes were due in part to the increased acetylation of these enzymes.

Metabolic flow in Nmnat3$^{gt/gt}$ erythrocytes was reversed to F1,6BP through the pentose phosphate pathway.

Because glycolysis enzymes in Nmnat3$^{gt/gt}$ erythrocytes showed increased activity compared with that in WT erythrocytes, we speculated that the inhibition of glycolysis pathway in Nmnat3$^{gt/gt}$ erythrocytes was due to the blockade

at GAPDH, which uses NAD as a coenzyme for the enzymatic reaction. Metabolomics data also suggested that the glycolysis pathway in Nmnat3$^{gt/gt}$ erythrocytes was stalled between F1,6BP and 3PG (Fig. 6A). However, we confirmed that the protein levels of GAPDH were unchanged in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6C). This finding also suggested that GAPDH was inhibited due to the reduced levels of NAD. To test this hypothesis, we performed *ex vivo* stable-isotope tracer analysis using 13C-labeled glucose. Erythrocytes isolated from WT and Nmnat3$^{gt/gt}$ mice were cultured in RPMI 1640 medium supplemented with 13C-labeled glucose, and samples were collected at several time points. First, [U-13C]-glucose was employed to evaluate the total flow of the glycolysis pathway. As shown in Fig. 7A, 13C-labeled F1,6BP increased much more rapidly in Nmnat3$^{gt/gt}$ erythrocytes. R5P/Ru5P and DHAP also increased more in Nmnat3$^{gt/gt}$ erythrocytes. However, the accumulation of downstream metabolites including GAP, 2,3-BPG, 3PG and PEP was clearly slower in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 7A). Thus, the glycolysis pathway in Nmnat3$^{gt/gt}$ erythrocytes was markedly slowed compared with that in WT erythrocytes.

From static metabolomics data, we observed that F1,6BP, but not GAP, was increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6A). [U-13C]-glucose tracer analysis also indicated that the flow became slower between F1,6BP and GAP, a step catalyzed by aldolase. However, the enzymatic activity of aldolase was not reduced in Nmnat3$^{gt/gt}$ erythrocytes (Fig. 6B). We accordingly hypothesized that the glucose flows from the pentose phosphate pathway was reversed between F1,6BP and GAP. To test this hypothesis, we used [1,2-13C]-glucose for further experiments to determine whether the flux to F1,6BP was directly from glycolysis or reversed from the pentose phosphate pathway via GAP. If F1,6BP were derived straightly from glucose, the 1,2-carbon (+2) of F1,6BP would be labeled. In contrast, if it was synthesized through the pentose phosphate pathway and reversed from GAP, the 1-carbon (+1), 1,2,4-carbon (+3) or 1,2,3,4-carbon (+4) of F1,6BP would be labeled with 13C (Fig. 7B). As shown in Fig. 7C, F1,6BP (+1), (+3) and (+4) were significantly increased in Nmnat3$^{gt/gt}$ erythrocytes, whereas the level of F1,6BP (+2) was near that of WT. Additionally, R5P/Ru5P (+1) was also increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig 7D). We concluded that the glycolytic flow in Nmnat3$^{gt/gt}$ erythrocytes was blocked at GAPDH owing to the shortage of NAD. Moreover, the glycolytic flow in Nmnat3$^{gt/gt}$ erythrocytes was shifted to the pentose phosphate pathway, and the blockade at GAPDH resulted in a reverse flow into F1,6BP from GAP.

Finally, we measured the ATP level of whole blood in the WT and Nmnat3$^{gt/gt}$ mice and found that the ATP level was markedly decreased in Nmnat3$^{gt/gt}$ mice (Fig. 7E). Thus, glycolysis blockade in Nmnat3$^{gt/gt}$ erythrocytes caused ATP production failure and ultimately lead to hemolytic anemia (Fig. 8).

DISCUSSION

Although the importance of NAD metabolism in various biological processes is known, the physiological function of Nmnat3 *in vivo* is not fully understood. In this study, we demonstrated that the NAD synthesis enzyme, Nmnat3, plays an essential role in the maintenance of the NAD pool in mature erythrocytes. Given that Nmnat3 is the dominant Nmnat among the three isoforms in mature erythrocytes, its deficiency leads to a drastic depletion of the NAD pool. Mature erythrocytes have no mitochondria, and their energy metabolism relies primarily on glycolysis. Thus, a lowered NAD level inhibits glycolysis at GAPDH and their ATP production is impaired in Nmnat3$^{gt/gt}$ erythrocytes. In erythrocytes, most ATP is consumed by Na^+-K^+-ATPase, a plasma membrane-embedded pump that maintains erythrocyte shape (48). An ATP shortage in Nmnat3$^{gt/gt}$ erythrocytes leads to dehydration of erythrocytes and a resulting spiked shape. These abnormally shaped erythrocytes are preferentially trapped and destroyed by the reticuloendothelial system of the spleen. Thus, Nmnat3 deficiency in mice caused splenomegaly and hemolytic anemia (Fig. 8).

Nmnat3$^{gt/gt}$ mice manifest hemolytic anemia from a very early age, as early as four weeks. It is well known that some of metabolic enzyme deficiencies can cause congenital hemolytic

anemia in humans (49). Nmnat3$^{gt/gt}$ mice exhibit a phenotype very similar to that of pyruvate kinase (PK) -deficient mice (50,51). Among PK isoforms, liver and RBC type PK (PKLR) deficiency cause hemolytic anemia. In PK-deficient mice, a shortened lifespan of mature erythrocytes primarily contributes to hemolytic anemia pathogenesis, whereas ineffective erythropoiesis also occurs in these mice (52). Thus, it is possible that ineffective erythropoiesis may occur in Nmnat3$^{gt/gt}$ mice. To date, no *Nmnat3* gene mutation has been found in human hemolytic anemia patients. However, hemolytic anemia in Nmnat3$^{gt/gt}$ mice resembles the phenotype of PK-deficient mice. Indeed, lack of PK in mice similarly causes the glycolysis inhibition and failure of ATP production. Notably, some hemolytic anemia patients clinically showed PK-deficiency-like hemolytic anemia, but neither enzymatic deficiency nor a genetic mutation in pyruvate kinase were not found (53). Thus, it may be that an *Nmnat3* gene mutation is hidden in human patients with congenital hemolytic anemia, particularly in undiagnosed PK deficiency-like patients.

We revealed that the level of NAD of Nmnat3$^{gt/gt}$ erythrocytes was drastically decreased compared with that of WT. In addition, mild decreases of NAM and NMN were also observed in Nmnat3$^{gt/gt}$ erythrocytes. One of the explanation of these decreases is that salvage pathway in Nmnat3$^{gt/gt}$ erythrocytes is impaired. In salvage pathway, NAM is supplied from NAD by sirtuin or PARP. It is uncertain that the conversion of NAM from NAD exists in mature erythrocytes. However, an extensive proteomic analysis identified PARP10, PARP12 and ADP-ribosylhydrolase 3 (ARH3) in human erythrocyte, as well as Nmnat3 (54). This fact implied the existence of salvage pathway by PARP in mature erythrocytes, but the actual molecular identities are still unknown. Another explanation for the reduction of NAM and NMN in Nmnat3$^{gt/gt}$ erythrocytes is that the incorporation of NAM was impaired in Nmnat3$^{gt/gt}$ erythrocytes due to the deformation of plasma membrane. Indeed, PRPP, the other substrate for NMN synthesis and Nampt, a NMN synthesis enzyme, were significantly increased in Nmnat3$^{gt/gt}$ erythrocytes (Fig 6A and Fig. 5K). These results also recapture the facts that NAM and NMN were decreased in Nmnat3$^{gt/gt}$ erythrocytes. However, molecular details of NAM incorporation into mature erythrocytes are still unclear and of interest for further investigations.

In Nmnat3$^{gt/gt}$ erythrocytes, F1,6BP was markedly increased due to the reverse flow from GAP. F1,6BP is known as an allosteric activator of pyruvate kinase (55). The accumulation of F1,6BP may increase the downstream flow of glycolysis by activating PK to compensate for the reduced glycolysis in Nmnat3$^{gt/gt}$ erythrocytes. We also demonstrated that the acetylation status in Nmnat3$^{gt/gt}$ erythrocytes was increased compared with that in WT. This difference could be due to decreased NAD and increased acetyl-CoA. As it is not verified whether sirtuin is present in erythrocytes, and thus the impact of decreased NAD in the hyper acetylation of Nmnat3$^{gt/gt}$ erythrocytes is unclear. However, a recent study suggests that protein acetylation in mitochondria occurs non-enzymatically and merely increased cellular acetyl-CoA can chemically acetylate proteins (56). It was reported that cytoplasmic acetyl-CoA in erythrocytes was generated from acetate by acetyl-CoA synthetase (57). It might be possible that the blockade of glycolysis in Nmnat3$^{gt/gt}$ erythrocytes indirectly activates the acetyl CoA synthesis pathway. We measured the activity of several glycolysis and pentose phosphate pathway enzymes and found their activities to be similar or increased compared with that in WT. These results suggested that acetylation increased, or at least did not decreases, the activity of these enzymes. We can accordingly speculate that acetylation is not the cause of glycolysis inhibition in Nmnat3$^{gt/gt}$ erythrocytes but a compensatory result for decreased glycolysis. Thus, the accumulation of F1,6BP and hyper acetylation of erythrocyte proteins would be a compensatory reaction for recovering the compromised ATP production in Nmnat3$^{gt/gt}$ erythrocytes.

Although Nmnat3 is considered a mitochondrial protein, our study revealed that Nmnat3 can resides in the cytoplasm of mature erythrocytes. A previous report also indicated that Nmnat3 enzymatic activity was dominant in human

erythrocytes (35). During the final step of erythropoiesis, mitochondria are excluded from reticulocytes. However, our result showed that Nmnat3 existed in the cytoplasm even in the reticulocytes. It was reported that some mitochondrial proteins are present in mature erythrocytes (54,58). Thus, it is desirable to investigate the subcellular localization of Nmnat3 in various cell types other than erythrocytes. In addition, Nmnat3 distribution is limited to certain tissues. If Nmnat3 is responsible for mitochondrial NAD synthesis, it should be present in most cell types that possess mitochondria. Further, we found that NAD levels in liver, skeletal muscle, and heart of Nmnat3$^{gt/gt}$ mice were similar to those in WT. Nmnat3$^{gt/gt}$ mice have no other phenotypes other than hemolytic anemia at normal housed condition, pointed out an intriguing question of how the mutant mice maintain their mitochondrial NAD level at Nmnat3 absence. It is currently accepted that NAD cannot cross the inner membrane of mitochondria and may be generated in the mitochondria matrix by Nmnat3 (59,60). However, our results suggest that NAD is imported into mitochondria through an unidentified NAD transporter. Indeed, other groups have proposed such an NAD transporter (61-63). We also showed that Nmnat3$^{gt/gt}$ mice have normal hematopoiesis and erythropoiesis. In Nmnat3$^{gt/gt}$ mice, white blood cell and platelets show normal numbers and subpopulations. The Nmnat3$^{gt/gt}$ erythroblast fraction has the same amount of NAD as WT. Our results accordingly suggest that Nmnat1 and Nmnat2 can compensate for mitochondrial NAD metabolism in these cells. As told, the mechanism of mitochondrial NAD supply may be more versatile than we understood currently.

In conclusion, our study has revealed the essential role of Nmnat3 on the NAD pool maintenance in mature erythrocytes. These findings provide new tools for studying the mechanism and pathophysiology of congenital hemolytic anemia and suggest the potential of NAD metabolism as a new therapeutic target of hemolytic anemia.

ACKNOWLEDGEMENT

We are grateful to Lee DH, Shinzawa K, and Chang HC for critical reading of the manuscript and Nakagawa Lab members for discussion. We also thank to Kazuyuki Nishio and Masahiko Kawahara (Life Science Research Center, University of Toyama) for helping the scanning electron microscopy experiments and Tomomi Kubo for mice care and husbandry.

REFERENCES

1. Chiarugi, A., Dolle, C., Felici, R., and Ziegler, M. (2012) The NAD metabolome--a key determinant of cancer cell biology. *Nat Rev Cancer* **12**, 741-752
2. Houtkooper, R. H., Canto, C., Wanders, R. J., and Auwerx, J. (2010) The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* **31**, 194-223
3. Guarente, L. (2013) Calorie restriction and sirtuins revisited. *Genes Dev* **27**, 2072-2085
4. Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., Yao, J., Zhou, L., Zeng, Y., Li, H., Li, Y., Shi, J., An, W., Hancock, S. M., He, F., Qin, L., Chin, J., Yang, P., Chen, X., Lei, Q., Xiong, Y., and Guan, K. L. (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science* **327**, 1000-1004
5. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z. B., Zeng, R., Xiong, Y., Guan, K. L., Zhao, S., and Zhao, G. P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* **327**, 1004-1007
6. Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004) Mammalian SIRT1 represses forkhead transcription factors. *Cell* **116**, 551-563

7. Purushotham, A., Schug, T. T., Xu, Q., Surapureddi, S., Guo, X., and Li, X. (2009) Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* **9**, 327-338
8. Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., and Puigserver, P. (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113-118
9. Mouchiroud, L., Houtkooper, R. H., Moullan, N., Katsyuba, E., Ryu, D., Canto, C., Mottis, A., Jo, Y. S., Viswanathan, M., Schoonjans, K., Guarente, L., and Auwerx, J. (2013) The NAD(+)/Sirtuin Pathway Modulates Longevity through Activation of Mitochondrial UPR and FOXO Signaling. *Cell* **154**, 430-441
10. Revollo, J. R., Korner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W., and Imai, S. (2007) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* **6**, 363-375
11. Yoshino, J., Mills, K. F., Yoon, M. J., and Imai, S. (2011) Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab* **14**, 528-536
12. Lau, C., Niere, M., and Ziegler, M. (2009) The NMN/NaMN adenylyltransferase (NMNAT) protein family. *Front Biosci (Landmark Ed)* **14**, 410-431
13. Berger, F., Lau, C., Dahlmann, M., and Ziegler, M. (2005) Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. *J Biol Chem* **280**, 36334-36341
14. Di Stefano, M., and Conforti, L. (2013) Diversification of NAD biological role: the importance of location. *FEBS J* **280**, 4711-4728
15. Chiang, P. W., Wang, J., Chen, Y., Fu, Q., Zhong, J., Chen, Y., Yi, X., Wu, R., Gan, H., Shi, Y., Chen, Y., Barnett, C., Wheaton, D., Day, M., Sutherland, J., Heon, E., Weleber, R. G., Gabriel, L. A., Cong, P., Chuang, K., Ye, S., Sallum, J. M., and Qi, M. (2012) Exome sequencing identifies NMNAT1 mutations as a cause of Leber congenital amaurosis. *Nat Genet* **44**, 972-974
16. Koenekoop, R. K., Wang, H., Majewski, J., Wang, X., Lopez, I., Ren, H., Chen, Y., Li, Y., Fishman, G. A., Genead, M., Schwartzentruber, J., Solanki, N., Traboulsi, E. I., Cheng, J., Logan, C. V., McKibbin, M., Hayward, B. E., Parry, D. A., Johnson, C. A., Nageeb, M., Finding of Rare Disease Genes Canada, C., Poulter, J. A., Mohamed, M. D., Jafri, H., Rashid, Y., Taylor, G. R., Keser, V., Mardon, G., Xu, H., Inglehearn, C. F., Fu, Q., Toomes, C., and Chen, R. (2012) Mutations in NMNAT1 cause Leber congenital amaurosis and identify a new disease pathway for retinal degeneration. *Nat Genet* **44**, 1035-1039
17. Perrault, I., Hanein, S., Zanlonghi, X., Serre, V., Nicouleau, M., Defoort-Delhemmes, S., Delphin, N., Fares-Taie, L., Gerber, S., Xerri, O., Edelson, C., Goldenberg, A., Duncombe, A., Le Meur, G., Hamel, C., Silva, E., Nitschke, P., Calvas, P., Munnich, A., Roche, O., Dollfus, H., Kaplan, J., and Rozet, J. M. (2012) Mutations in NMNAT1 cause Leber congenital amaurosis with early-onset severe macular and optic atrophy. *Nat Genet* **44**, 975-977
18. Falk, M. J., Zhang, Q., Nakamaru-Ogiso, E., Kannabiran, C., Fonseca-Kelly, Z., Chakarova, C., Audo, I., Mackay, D. S., Zeitz, C., Borman, A. D., Staniszewska, M., Shukla, R., Palavalli, L., Mohand-Said, S., Waseem, N. H., Jalali, S., Perin, J. C., Place, E., Ostrovsky, J., Xiao, R., Bhattacharya, S. S., Consugar, M., Webster, A. R., Sahel, J. A., Moore, A. T., Berson, E. L., Liu, Q., Gai, X., and Pierce, E. A. (2012) NMNAT1 mutations cause Leber congenital amaurosis. *Nat Genet* **44**, 1040-1045
19. Mack, T. G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L., Fernando, F. S., Tarlton, A., Andressen, C., Addicks, K., Magni, G., Ribchester, R. R., Perry, V. H., and Coleman, M. P. (2001) Wallerian

degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat Neurosci* **4**, 1199-1206

20. Sasaki, Y., Vohra, B. P., Baloh, R. H., and Milbrandt, J. (2009) Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. *J Neurosci* **29**, 6526-6534
21. Conforti, L., Janeckova, L., Wagner, D., Mazzola, F., Cialabrini, L., Di Stefano, M., Orsomando, G., Magni, G., Bendotti, C., Smyth, N., and Coleman, M. (2011) Reducing expression of NAD+ synthesizing enzyme NMNAT1 does not affect the rate of Wallerian degeneration. *FEBS J* **278**, 2666-2679
22. Gilley, J., and Coleman, M. P. (2010) Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. *PLoS Biol* **8**, e1000300
23. Yan, T., Feng, Y., Zheng, J., Ge, X., Zhang, Y., Wu, D., Zhao, J., and Zhai, Q. (2010) Nmnat2 delays axon degeneration in superior cervical ganglia dependent on its NAD synthesis activity. *Neurochem Int* **56**, 101-106
24. Hicks, A. N., Lorenzetti, D., Gilley, J., Lu, B., Andersson, K. E., Miligan, C., Overbeek, P. A., Oppenheim, R., and Bishop, C. E. (2012) Nicotinamide mononucleotide adenylyltransferase 2 (Nmnat2) regulates axon integrity in the mouse embryo. *PLoS One* **7**, e47869
25. Gilley, J., Adalbert, R., Yu, G., and Coleman, M. P. (2013) Rescue of peripheral and CNS axon defects in mice lacking NMNAT2. *J Neurosci* **33**, 13410-13424
26. Nikiforov, A., Dolle, C., Niere, M., and Ziegler, M. (2011) Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. *J Biol Chem* **286**, 21767-21778
27. Sasaki, Y., Araki, T., and Milbrandt, J. (2006) Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. *J Neurosci* **26**, 8484-8491
28. Yahata, N., Yuasa, S., and Araki, T. (2009) Nicotinamide mononucleotide adenylyltransferase expression in mitochondrial matrix delays Wallerian degeneration. *J Neurosci* **29**, 6276-6284
29. Preiss, J., and Handler, P. (1957) Enzymatic synthesis of nicotinamide mononucleotide. *J Biol Chem* **225**, 759-770
30. Micheli, V., and Sestini, S. (1997) Determining NAD synthesis in erythrocytes. *Methods Enzymol* **280**, 211-221
31. Micheli, V., Simmonds, H. A., Sestini, S., and Ricci, C. (1990) Importance of nicotinamide as an NAD precursor in the human erythrocyte. *Arch Biochem Biophys* **283**, 40-45
32. Micheli, V., Sestini, S., Rocchigiani, M., Pescaglini, M., and Ricci, C. (1991) Significance and relevance of NAD synthesis in human erythrocyte life span. *Adv Exp Med Biol* **307**, 139-146
33. Sestini, S., Ricci, C., Micheli, V., and Pompucci, G. (1993) Nicotinamide mononucleotide adenylyltransferase activity in human erythrocytes. *Arch Biochem Biophys* **302**, 206-211
34. Liu, R., and Orgel, L. E. (1995) Enzymatic synthesis of polymers containing nicotinamide mononucleotide. *Nucleic Acids Res* **23**, 3742-3749
35. Di Stefano, M., Galassi, L., and Magni, G. (2010) Unique expression pattern of human nicotinamide mononucleotide adenylyltransferase isozymes in red blood cells. *Blood Cells Mol Dis* **45**, 33-39
36. Noble, N. A., Xu, Q. P., and Ward, J. H. (1989) Reticulocytes. I. Isolation and in vitro maturation of synchronized populations. *Blood* **74**, 475-481
37. Manodori, A. B., and Kuypers, F. A. (2002) Altered red cell turnover in diabetic mice. *J Lab Clin Med* **140**, 161-165
38. Orsomando, G., Cialabrini, L., Amici, A., Mazzola, F., Ruggieri, S., Conforti, L., Janeckova, L., Coleman, M. P., and Magni, G. (2012) Simultaneous single-sample determination of

NMNAT isozyme activities in mouse tissues. *PLoS One* **7**, e53271

39. Beutler, E., Blume, K. G., Kaplan, J. C., Lohr, G. W., Ramot, B., and Valentine, W. N. (1977) International Committee for Standardization in Haematology: recommended methods for red-cell enzyme analysis. *Br J Haematol* **35**, 331-340
40. Yamada, K., Hara, N., Shibata, T., Osago, H., and Tsuchiya, M. (2006) The simultaneous measurement of nicotinamide adenine dinucleotide and related compounds by liquid chromatography/electrospray ionization tandem mass spectrometry. *Anal Biochem* **352**, 282-285
41. Darghouth, D., Koehl, B., Madalinski, G., Heilier, J. F., Bovee, P., Xu, Y., Olivier, M. F., Bartolucci, P., Benkerrou, M., Pissard, S., Colin, Y., Galacteros, F., Bosman, G., Junot, C., and Romeo, P. H. (2011) Pathophysiology of sickle cell disease is mirrored by the red blood cell metabolome. *Blood* **117**, e57-66
42. Sporty, J. L., Kabir, M. M., Turteltaub, K. W., Ognibene, T., Lin, S. J., and Bench, G. (2008) Single sample extraction protocol for the quantification of NAD and NADH redox states in Saccharomyces cerevisiae. *J Sep Sci* **31**, 3202-3211
43. Yuan, M., Breitkopf, S. B., Yang, X., and Asara, J. M. (2012) A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat Protoc* **7**, 872-881
44. Felici, R., Lapucci, A., Ramazzotti, M., and Chiarugi, A. (2013) Insight into Molecular and Functional Properties of NMNAT3 Reveals New Hints of NAD Homeostasis within Human Mitochondria. *PLoS One* **8**, e76938
45. van den Bos, C., Kieboom, D., Visser, T. P., and Wagemaker, G. (1993) Compensatory splenic hemopoiesis in beta-thalassemic mice. *Exp Hematol* **21**, 350-353
46. Loken, M. R., Shah, V. O., Dattilio, K. L., and Civin, C. I. (1987) Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood* **69**, 255-263
47. Stanton, R. C. (2012) Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* **64**, 362-369
48. Liu, J., Mohandas, N., and An, X. (2011) Membrane assembly during erythropoiesis. *Curr Opin Hematol* **18**, 133-138
49. Climent, F., Roset, F., Repiso, A., and Perez de la Ossa, P. (2009) Red cell glycolytic enzyme disorders caused by mutations: an update. *Cardiovasc Hematol Disord Drug Targets* **9**, 95-106
50. Kanno, H., Morimoto, M., Fujii, H., Tsujimura, T., Asai, H., Noguchi, T., Kitamura, Y., and Miwa, S. (1995) Primary structure of murine red blood cell-type pyruvate kinase (PK) and molecular characterization of PK deficiency identified in the CBA strain. *Blood* **86**, 3205-3210
51. Morimoto, M., Kanno, H., Asai, H., Tsujimura, T., Fujii, H., Moriyama, Y., Kasugai, T., Hirono, A., Ohba, Y., Miwa, S., and Kitamura, Y. (1995) Pyruvate kinase deficiency of mice associated with nonspherocytic hemolytic anemia and cure of the anemia by marrow transplantation without host irradiation. *Blood* **86**, 4323-4330
52. Aizawa, S., Harada, T., Kanbe, E., Tsuboi, I., Aisaki, K., Fujii, H., and Kanno, H. (2005) Ineffective erythropoiesis in mutant mice with deficient pyruvate kinase activity. *Exp Hematol* **33**, 1292-1298
53. Miwa, S., Kanno, H., and Fujii, H. (1993) Concise review: pyruvate kinase deficiency: historical perspective and recent progress of molecular genetics. *Am J Hematol* **42**, 31-35
54. Roux-Dalvai, F., Gonzalez de Peredo, A., Simo, C., Guerrier, L., Bouyssie, D., Zanella, A., Citterio, A., Burlet-Schiltz, O., Boschetti, E., Righetti, P. G., and Monsarrat, B. (2008) Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol Cell Proteomics* **7**, 2254-2269

55. Kirtley, M. E., and McKay, M. (1977) Fructose-1,6-bisphosphate, a regulator of metabolism. *Mol Cell Biochem* **18**, 141-149
56. Wagner, G. R., and Payne, R. M. (2013) Widespread and enzyme-independent Nepsilon-acetylation and Nepsilon-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *J Biol Chem* **288**, 29036-29045
57. Pittman, J. G., and Martin, D. B. (1966) Fatty acid biosynthesis in human erythrocytes: evidence in mature erythrocytes for an incomplete long chain fatty acid synthesizing system. *J Clin Invest* **45**, 165-172
58. Tyan, Y. C., Jong, S. B., Liao, J. D., Liao, P. C., Yang, M. H., Liu, C. Y., Klauser, R., Himmelhaus, M., and Grunze, M. (2005) Proteomic profiling of erythrocyte proteins by proteolytic digestion chip and identification using two-dimensional electrospray ionization tandem mass spectrometry. *J Proteome Res* **4**, 748-757
59. Stein, L. R., and Imai, S. (2012) The dynamic regulation of NAD metabolism in mitochondria. *Trends Endocrinol Metab* **23**, 420-428
60. Dolle, C., Rack, J. G., and Ziegler, M. (2013) NAD and ADP-ribose metabolism in mitochondria. *FEBS J* **280**, 3530-3541
61. Haferkamp, I., Schmitz-Esser, S., Linka, N., Urbany, C., Collingro, A., Wagner, M., Horn, M., and Neuhaus, H. E. (2004) A candidate NAD+ transporter in an intracellular bacterial symbiont related to Chlamydiae. *Nature* **432**, 622-625
62. Palmieri, F., Rieder, B., Ventrella, A., Blanco, E., Do, P. T., Nunes-Nesi, A., Trauth, A. U., Fiermonte, G., Tjaden, J., Agrimi, G., Kirchberger, S., Paradies, E., Fernie, A. R., and Neuhaus, H. E. (2009) Molecular identification and functional characterization of Arabidopsis thaliana mitochondrial and chloroplastic NAD+ carrier proteins. *J Biol Chem* **284**, 31249-31259
63. Song, E. K., Rah, S. Y., Lee, Y. R., Yoo, C. H., Kim, Y. R., Yeom, J. H., Park, K. H., Kim, J. S., Kim, U. H., and Han, M. K. (2011) Connexin-43 hemichannels mediate cyclic ADP-ribose generation and its Ca2+-mobilizing activity by NAD+/cyclic ADP-ribose transport. *J Biol Chem* **286**, 44480-44490

FOOTNOTES

This work is supported by a Grant-in-Aid for Young Scientists (A) (24609019) and a Grant-in-Aid for Scientific Research on Innovative Areas (24116511) to T.N. This work is also supported by a grant from The Sumitomo Foundation, a grant from Kanae Foundation for the Promotion of Medical Science, a grant from Astellas Foundation for Research on Metabolic Disorders, and a grant from Takeda Science Foundation to T.N. The authors declare no competing financial interests.