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Targeted Deletion of the γ-Adducin Gene (*Add3*) in Mice Reveals Differences in α-Adducin Interactions in Erythroid and Nonerythroid Cells

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Abstract

In red blood cells (RBCs) adducin heterotetramers localize to the spectrin-actin junction of the peripheral membrane skeleton. We previously reported that deletion of β-adducin results in osmotically fragile, microcytic RBCs and a phenotype of hereditary spherocytosis (HS). Notably, α-adducin was significantly reduced, while γ-adducin, normally present in limited amounts, was increased ~5-fold, suggesting that α-adducin requires a heterologous binding partner for stability and function, and that γ-adducin can partially substitute for the absence of β-adducin. To test these assumptions we generated γ-adducin null mice. γ-adducin null RBCs appear normal on Wright's stained peripheral blood smears and by scanning electron microscopy. All membrane skeleton proteins examined are present in normal amounts, and all hematological parameters measured are normal. Despite a loss of ~70% of α-adducin in γ-adducin null platelets, no bleeding defect is observed and platelet structure appears normal. Moreover, systemic blood pressure and pulse are normal in γ-adducin null mice. γ- and β-adducin null mice were intercrossed to generate double null mice. Loss of γ-adducin does not exacerbate the β-adducin null HS phenotype although the amount α-adducin is reduced to barely detectable levels. The stability of α-adducin in the absence of a heterologous binding partner varies considerably in various tissues. The amount of α-adducin is modestly reduced (~15%) in the kidney, while in the spleen and brain is reduced by ~50% with the loss of a heterologous β- or γ-adducin binding partner. These results suggest that the structural properties of adducin differ significantly between erythroid and various nonerythroid cell types.

INTRODUCTION

The adducins are a family of three closely related cytoskeletal proteins (α, β and γ) encoded by distinct genes (*Add1*, *Add2*, and *Add3*, respectively)1. Two or more adducins are expressed in all mouse and human tissues examined[2]. In red blood cells (RBCs) where it was first purified as a calmodulin-binding protein, adducin is comprised of α- and β-polypeptides that tightly interact in a 1:1 stoichiometry to form heterodimers and heterotetramers[3]. Adducin isolated from the kidney on the basis of its protein kinase C binding activity is comprised of α- and γ-polypeptides4. While the α- and γ-adducin genes are expressed in all mouse tissues examined, β-adducin gene expression is more restricted with highest levels in erythroid cells and the brain2. All three adducin genes are alternatively spliced1.

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In RBCs, where its function has been most studied, adducin is localized to the spectrin-actin junctional complex of the peripheral membrane skeleton5. Other components of this complex include tropomodulin, tropomyosin, proteins 4.1 and 4.9 (dematin), and p55. The interactions of these proteins play critical roles in the assembly of the membrane skeleton during red cell maturation and in the maintenance of mature red cell shape and deformability6. Adducin has been shown to: (1) regulate the length of actin filaments by capping the fast growing barbed end7,8; (2) bundle actin filaments9; and (3) recruit spectrin to the fast growing ends of actin filaments10. The functional properties of adducin are modulated by calmodulin and phosphorylation by PKA, PKC, and Rho-associated kinase8,11-13. Because of these features, adducin has been proposed to play a crucial role in assembly and stability of the RBC membrane skeleton6. In support of this hypothesis we have previously shown that loss of β-adducin results in osmotically fragile, microcytic RBCs, and an overall phenotype of hereditary spherocytosis (HS)[2]. Strikingly, the absence of β-adducin was found to reduce the relative amount of α-adducin in RBCs to ~30% of normal. A surprising result of this study was the detection of γ-adducin in the membrane skeleton of normal mouse red blood cells, which was significantly increased (~5-fold) in ghosts prepared from β-adducin null RBCs. (γ-adducin has not been detected in human RBCs.) These findings suggest that in the mouse RBC membrane skeleton stable, functional adducin requires the presence of at least two heterologous subunits, primarily α- and β-polypeptides, and that up-regulation of γ-adducin can partially compensate for the absence of β-adducin.

Adducin maintains the shape of resting platelets and participates in platelet activation as a substrate for protein kinase C and calpain signaling14. Adducin has also been implicated in the control of systemic blood pressure in humans and in animal models15-18. A single α-adducin amino acid change (F→Y) is associated with a hypertensive phenotype in the Milan hypertensive rat strain (MHS), while a segregating β-adducin amino acid substitution (Q→R) modulates the hypertensive phenotype[19]. Hypertension has also been reported in β-adducin null mice20.

In an effort to analyze the function of γ-adducin in greater detail, we have generated a γ-adducin knockout in mice using a Cre-loxP strategy. Phenotypically, γ-adducin null mice display normal growth characteristics and show no overt defects. The loss of γ-adducin alone does not significantly impact RBC membrane skeleton structure or RBC function, platelet structure or function, or systemic blood pressure. The additional loss of γ-adducin does not exacerbate the β-adducin null HS phenotype. The amount of α-adducin in the β/γ-adducin double null RBC membrane skeleton, however, is dramatically reduced to barely detectable levels (<1% of normal), suggesting that the function and stability of RBC adducin depends critically on the ability to form heterotetramers. In platelets the loss of γ-adducin results in a ~70% loss of α-adducin. In contrast, the loss of the γ-adducin subunit in the kidney results in only a modest loss (~15%) of α-adducin, while in the spleen and brain loss of β- and γ-adducin heterologous binding subunits results in a ~50% reduction in the amount of α-adducin. These results suggest that the structural properties of adducin differ significantly between erythroid and nonerythroid cells, and that α-adducin may function as a homodimer or homotetramer and/or be stabilized by interaction with other proteins in some cell types.

RESULTS

γ-Adducin Gene Targeting

We used a *loxP* strategy to delete exon 2, the first protein coding exon, of the γ-adducin gene (Fig 1). The effectiveness of gene targeting was assessed by northern and western analysis of wild type (wt) (+/+), heterozygous (+/−), and homozygous null (−/−) offspring of intercrosses of γ-add$^{\Delta ex2\Delta neo}$/+ mice, generated as described in detail in Methods. By northern analysis of spleen total RNA, a cDNA probe specific for γ-adducin gene exon 2 detected a major transcript

of ~4 kb in RNA isolated from +/+ and +/− littermates, but failed to detect a transcript in RNA isolated from a −/− littermate (Fig. 1F). Surprisingly, a γ-adducin cDNA probe corresponding to exons 6-10 detected a ~4 kb transcript in +/+, +/− and in −/− spleen RNA. The presence of a γ-adducin transcript in the −/− sample is likely the result of abnormal splicing of the γ-add$^{\Delta ex2\Delta neo}$ pre-mRNA resulting in a mature transcript lacking sequences encoded by exon 2. The small difference in the expected size of this transcript compared to the wt transcript[1,2], only 215 bp, could not be detected by northern analysis, but was confirmed by RT-PCR (not shown). The absence of γ-adducin protein in the spleens of −/− mice, however, was directly shown by western analysis (Fig. 1F insert). The antibody used is directed against amino acid residues 571-630 at the C-terminus of human γ-adducin, a region of the mouse protein encoded by exons 13-15. As shown in Fig. 1F, protein isolated from +/+ and +/− spleens have two products corresponding to the 674 and 706 amino acid γ-1 and γ-2 adducin polypeptides previously described[1]. Both of these products are absent in the sample prepared from the −/− spleen. Notably, both α- and β-adducin proteins are present at normal levels in the absence of γ-adducin (Fig. 1F).

Intercrossing heterozygous mice (γ-add$^{\Delta ex2\Delta neo}$/+) yielded, in the expected Mendelian ratios, homozygous null mice that were overtly indistinguishable from their +/+ and +/− littermates.

Loss of γ-Adducin Does Not Alter Red Cell Structure/Function or Exacerbate the β-Adducin Null HS Phenotype

Although adducin in mouse red blood cells is predominantly in the form of α/β-oligomers[5,21], a relatively small amount of γ-adducin is also present[2], which is presumably in the form of α/γ- heterodimers and heterotetramers. Red blood cells isolated from γ-adducin null mice are normal in overall size and shape when examined by either bright field microscopy or by SEM (Fig. 2). Comparison of hematological values (Table 2), osmotic fragility (Fig. 3A), and ektacytometry (Fig. 3B) revealed no significant differences between wt and γ-adducin null red cells. All of the membrane skeleton proteins examined by SDS/PAGE and western blotting, including α- and β-adducin, spectrin, ankyrin, band 3, protein 4.1, protein 4.2, protein 4.9 (dematin), tropomodulin (Tmod), tropomyosin (TM), and actin are present in normal amounts in red cell ghosts prepared from γ-adducin mice (Fig. 1F, 4A).

As previously described[2], deletion of β-adducin in mice results in a greater number of smaller red blood cells with a variety of shapes including spherocytes, spherostomatocytes, and rounded elliptocytes, with an overall phenotype of HS. The hematocrit of these mice compared to wt mice was significantly decreased (47.8% vs 51.4%), while the reticulocyte percentage was significantly increased (4.3% vs 2.8%). The finding of a ~5-fold increase in the amount of γ-adducin present in the membrane skeleton of these red cells suggests that this adducin subunit may be able to form stable heterotetramers with α-adducin and partially compensate for the loss of β-adducin. To test this, mice null for both β- and γ-adducin were produced by intercrossing β- and γ-adducin +/− mice. β/γ-adducin null mice were produced in the expected numbers. The additional loss of γ-adducin does not exacerbate the β-adducin null red cell HS phenotype. Analysis by bright field microscopy and SEM (Fig. 2) reveals a similar number of small and abnormally shaped cells in blood isolated from β- or β/γ-adducin null mice. Advia analysis (Table 2) reveals near identical RBC counts, HCT, MCV, MCH, MCHC, and reticulocyte percentages in red blood cells of β- and β/γ-adducin null mice. Fragility of β- and β/γ-adducin null red blood cells (Fig. 3) is also increased to the same degree compared to +/+ red blood cells.

The length of actin filaments is closely regulated. In RBCs this is accomplished primarily by adducin and tropomodulin which have been shown to cap the fast growing barbed end and the slow growing ends of actin filaments, respectively, and by tropomyosin, a rod-like protein that interacts along the length of the action filament[10,22,23]. As previously reported[24], loss of β-

adducin results in significant increases in the amounts of membrane-associated EcapZ-α and EcapZ-β. Normally, these two actin barbed end capping proteins do not appear to participate in regulating the length of actin filaments of the red cell membrane skeleton but are restricted to the cytoplasm[7]. In the absence of β-adducin, however, both become membrane-associated[24]. These authors also report a decrease in the amount of tropomyosin. We observe similar changes in the β-adducin null RBC membrane skeleton (Fig. 4A). The loss of γ-adducin alone does not change the relative amounts of these components in the RBC membrane skeleton compared to wild type cells, and the additional loss of γ-adducin does not exacerbate their changes caused by the loss of β-adducin (Fig. 4A).

Combined Loss of β- and γ-adducin Prevents Stable Incorporation of α-adducin into the Red Cell Membrane Skeleton

Red cell ghosts isolated from β-adducin null mice have significantly reduced amounts of α-adducin and increased amounts of γ-adducin[2], suggesting that the stable incorporation of adducin into the RBC membrane skeleton requires heterologous binding partners, normally α- and β-adducin, and that α- and γ-adducin can interact and partially compensate for the loss of the β-adducin. These possibilities and whether α-adducin can function alone were examined directly by comparing red blood cells isolated from wt, β-adducin null, and β/γ-adducin double null mice. As previously reported[2], the amount of α-adducin is dramatically decreased by the loss of β-adducin (Fig. 4B, lane 6), in this experiment to ~20% of wt levels. Significantly, the relative amount of α-adducin in β/γ-null red cell ghost preparations is further reduced to <1% of wt (Fig. 4B, lane 8). The membrane skeleton of γ-adducin null RBCs shows normal amounts of α- and β-adducin (Fig. 4B, lane 7). These results are consistent with the idea that stable incorporation of adducin into the red cell membrane skeleton requires the formation of hetero-oligomers, and that α-adducin homo-oligomers do not form or are not stable.

Loss of γ-adducin in Platelets Results in Significant Loss of α-adducin

As shown in Fig. 5A (lanes 3 and 4), ~70% of α-adducin is lost in platelets of γ-adducin null mice. As previously reported in human platelets[2], we did not detect any β-adducin in mouse platelets (not shown). Advia analysis (Table 2) revealed no significant changes in mean platelet volume (MPV) or in platelet numbers, and γ-adducin null and wt platelets have similar structure and shape by transmission electron microscopy (Fig. 5C). The bleeding times of γ-adducin null and wt mice were similar (Fig. 5B), and both have similar whole blood aggregation times (data not shown). These results are similar to those we have previously observed in α-adducin null mice[25]. Thus, although adducin maintains the shape of resting platelets and participates in platelet activation as a substrate for protein kinase C and calpain signaling in vitro14, we find no evidence of major platelet dysfunction in the absence of adducin in vivo.

Blood Pressure Is Normal in γ-adducin Null Female Mice

We backcrossed γ-adducin null mice to the inbred strain 129S1/SvImJ for 6 generations and measured systolic, diastolic and mean arterial pressure and pulse rate in γ-null and wt females. No differences in any of these parameters were seen (Table 3).

α-adducin Is Comparatively Stable in Nonerythroid Cells in the Absence of β- and γ-adducin

In nonerythroid cells adducin is localized to the plasma membrane in association with spectrin and actin, and concentrated at the sites of cell-cell contact6. While all three adducin subunits are expressed in the brain and spleen, in other nonerythroid cell types adducin is comprised of α- and γ-polypeptides[2]. The severe loss of α-adducin in β/γ–adducin double null RBCs, described above, suggests that stable incorporation of adducin into the peripheral membrane skeleton in RBCs requires the formation of α/β or α/γ heterotetramers. To test if this is also the case in other tissues, the relative amounts of the adducin polypeptides in platelets (described

above), spleen, kidney, and brain tissues of wt, β null, γ null, and β/γ null mice were compared by western blotting. In the spleen the loss of β-adducin or γ-adducin alone does not significantly affect the levels of α-adducin (Fig. 6, lanes 4, 6). The loss of β- and γ-adducin together, however, reduces α-adducin to ~50% of normal (Fig. 6, lane 8). This is significantly more than we see in terminally differentiated RBC ghosts, where α-adducin levels are reduced to barely detectable levels, suggesting that considerably more α-adducin in retained in either erythroid progenitors or white blood cells, or both.

In the brain, loss of β-adducin alone decreases α-adducin membrane content modestly (~10%) (Fig. 6, lane 20), while loss of γ-adducin alone has no effect (Fig. 6, lane 22). The combined loss of β- and γ-adducin in the brain impacts α-adducin levels significantly, reducing it to ~50% of normal (Fig. 6, lane 24), as in the spleen. Notably, in the kidney, there is only a modest decrease (~15%) in the amount of α-adducin in the absence of β-adducin (lane 12), γ-adducin (lane 14), or the combined loss of β- and γ-adducin (lane 16).

DISCUSSION

The adducins are a family of three closely related cytoskeletal proteins (α, β and γ) encoded by distinct genes[1]. The amino acid sequences and potential structural domains of all three adducin polypeptides are very similar to each other and highly conserved in various species1‚6. Each polypeptide subunit consists of an N-terminal globular head domain, a neck domain, and a C-terminal protease-sensitive region containing a MARCKS-related domain6. In RBCs where its structure and function are best understood, functional adducin is primarily in the form of heterotetramers[5,6] While the predominant species is the α/β form, the small amount of γ-adducin found in mouse RBCs is presumably in the form of α/γ heterotetramers based upon observations in the β-adducin null mouse[2]. The currently held view is that formation of hetero-oligomers is required for red cell adducin structure and function since no monomeric adducin has been observed *in vivo*[6]. Our results support this model since the loss of β-adducin results in a dramatic loss of the α-subunit, and the combined loss of β- and γ-adducin greatly exacerbates its loss from the peripheral membrane skeleton of the red cell. In agreement with this is the recent finding that loss of α-adducin from the RBC membrane skeleton in α-adducin null mice also results in the complete absence of both β- and γ-adducin polypeptides[26].

During red cell maturation and adducin oligomer formation, β- and γ-adducin polypeptides, the latter made only in relatively small amounts, compete for binding to limiting amounts of α-adducin. Because of this competitive process, the absence of β-adducin would allow more γ-adducin to form heterodimers and heterotetramers with α-adducin resulting in the ~5-fold increase in γ-adducin that we observe in β-adducin null RBCs. We suggest that this relatively large increase is not able to fully stabilize α-adducin and correct the HS phenotype either because α/β- and α/γ-adducin oligomers are not structurally and functionally equivalent, or because not enough γ-adducin is made to fully occupy the available α-adducin subunits. Experiments to test these possibilities by overexpressing γ-adducin in β-adducin null RBCs are underway.

While the loss of β-adducin has a significant impact on the mouse RBC, resulting in osmotically fragile, microcytic RBCs, with an overall phenotype of HS[2], the loss of γ-adducin has no obvious structural or functional consequences. These RBCs are normal in overall size and shape when examined by either bright field microscopy or by scanning electron microscopy, all hematological values measured are normal, and other components of the membrane skeleton do not appear to be affected by the loss of γ-adducin. We also find that the additional loss of γ-adducin does not exacerbate the β-adducin null red cell HS phenotype. The findings described in this report and previously published[2,25] indicate that adducin in RBCs is primarily in the

form of α/β-heterodimers and heterotetramers, and that γ-adducin plays a limited role in the RBC membrane skeleton structure and function.

In blood platelets, loss of γ-adducin results in a significant loss of α-adducin (~70%) but has no apparent affect on platelet structure, number, or function as determined by bleeding time analysis and whole blood aggregation rates. We have described similar results in α-adducin null mice[25].

Two or more adducins are expressed in all mouse and human tissues examined[2]. While α- and γ-adducin genes are expressed in a ubiquitous manner, β-adducin gene expression is restricted to erythroid cells and the brain. In this report we asked directly if α-adducin can function alone or if it requires a heterologous β- or γ-adducin binding partner. Our results show that there are striking tissue-specific differences. In RBCs adducin stability requires heterologous binding partners since the combined loss of β- and γ-adducin leads to a near complete loss of α-adducin from the red cell membrane skeleton. In platelets, loss of γ-adducin results in a significant loss (~70%) of α-adducin. In other nonerythroid tissues analyzed, however, our results suggest that α-adducin is considerably more stable in the absence of a heterologous β- or γ-adducin binding partner. It is not known whether this is the result of the ability of α-adducin to form homo-oligomers, or due to its being stabilized by interaction with other membrane components. Although adducin homo-oligomers have not been observed *in vivo*, recombinant adducin polypeptides are capable of forming homodimers *in vitro*[5]. In *C. elegans* and *D. melanogaster* adducin is encoded by a single gene and the adducin protein presumably exists in either the homodimeric or homotetrameric form.[5] In the kidney[27], α- and γ-adducin have been reported to be localized to the apical and basal membranes of proximal tubules, respectively, suggesting that these adducin polypeptides may have distinct structural and functional properties, and do not require the formation of hetero-oligomers.

We recently showed that lethal communicating hydrocephalus occurs in ~50% of α-adducin null mice on the B6, 129 segregating background [25]. In this study, no evidence of hydrocephalus is seen in β-, γ- or βγ-adducin null mice maintained on the same genetic background. As α-adducin is completely lacking in the brain in our previously described α-adducin knockout, but not in β-, γ- or βγ-adducin null brain, it is clear that α-adducin plays a critical role in cerebrospinal fluid homeostasis.

In summary: (1) Because the loss of γ-adducin does not significantly impact RBC structure or function or exacerbate the β-adducin null HS phenotype, γ-adducin probably plays a limited role in RBC membrane skeleton structure and function in the mouse. The principle adducin in RBCs is in the form of α/β-oligomers. (2) In platelets loss of γ-adducin results in a significant loss of α-adducin but no impairment of platelet function. (3) In erythroid and other nonerythroid tissues, α- and β-adducin are stable and present at relatively normal levels in the absence of γ-adducin. (4) In RBCs the formation of hetero-oligomers is required for adducin stability and function since α-adducin is lost from the membrane skeleton of β/γ-adducin double null RBCs, and β- and γ-adducin are lost from the membrane skeleton of α-adducin null RBCs[26]. (5) In spleen, kidney, and brain α-adducin appears to be comparatively more stable than in RBCs in the absence of either β- or γ-adducin binding partners, suggesting that the structure and function of adducin varies significantly between RBCs and these nonerythroid tissues.

METHODS

Animals

Mice were maintained at The Jackson Laboratory in a climate-controlled room with 12 hour light cycles and were provided acidified water and chow (NIH 5K52) *ad libitum*. All procedures were approved by The Jackson Laboratory Animal Care and Use Committee.

Targeted Disruption of the Mouse γ-adducin (Add3) Gene

Briefly, a ~12 kb mouse γ-adducin gene fragment (Fig. 1A) containing the first protein coding exon (gene exon 2) and flanking upstream and downstream intron regions was isolated from a 129/Sv λFixII library (Stratagene, La Jolla, CA) by standard methods[28], and subcloned into the pBSK vector (Invitrogen). (γ-adducin gene exon/intron organization is according to Ensemble transcript ENSMUST00000111741.) To make a conditional targeting construct, *loxP* sequences were inserted ~1.6 kb upstream and ~0.4 kb downstream of exon 2, into *Eco*RV and *Afe*I restriction sites, respectively. A neo^r gene flanked by FRT sequences[29] was inserted immediately downstream of the intron 2 *loxP* sequence. After linearization with *Not*I, 129P2/OlaHsd-derived E14 ES cells were transfected by electroporation and G418^r clones were isolated. Correctly targeted clones were identified by Southern hybridization[28, 30] to purified genomic DNA using an upstream probe following digestion by *Nde*I and *Xho*I, and using a downstream probe following digestion by *Xba*I. A correctly targeted ES cell subclone (1A10) was used for blastocyst injection and embryo transfer[31]. One high chimera male was obtained and mated to a C57BL/6J female to generate heterozygous targeted mice (γ-add^{CKO}/+), initially identified by neo^r gene PCR, and confirmed by 5′ and 3′ Southern analysis of tail DNA (Fig. 1B,C), as described above. The neo^r gene was excised by mating a correctly targeted heterozygous mouse to a homozygous *Flp* mouse[29] (strain:129S4/SvJaeSor-*Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$*/J; Jackson Laboratory Stock Number 003946); γ-add$^{\Delta neo}$/+ pups were identified by PCR (Fig. 1D). The floxed exon 2 was then excised by mating a γ-add$^{\Delta neo}$/+ mouse to a homozygous EIIa Cre mouse[32] (strain:B6.FVB-Tg(EIIa-cre) C5379Lmgd/J; Jackson Laboratory Stock Number 003724), producing heterozygous mice (γ-add$^{\Delta ex2\Delta neo}$/+) with the targeted γ-adducin allele lacking both exon 2 and the neo^r cassette (Fig. 1E).

For PCR genotyping, the following primers were used: γ-addf1 (5′-aggcccaccatgcacctcaccggca) and γ-addr1 (5′-accttccaagctgaccaata) flank the location of the upstream *loxP* sequence and generate 239 bp and 275 bp wild type (wt) and targeted products, respectively; γ-addf2 (5′-caggatgctgagaacttgtg) and γ-addr2 (5′-tgacagtagcaagatcacag) flank the location of the intron 2 FRT-neo^r sequence and generate 275 bp, 400 bp, and 1500 bp wt, Δneo, and neo^r products, respectively. A MasterTaq kit (Eppendorf, Hamburg, Germany) was used for PCR amplification.

Isolation of RNA and Northern Analysis

Total RNA was isolated from the spleens of adult mice using TRIzol Reagent (Invitrogen) and analyzed by northern hybridization using standard methods[28]. For probe synthesis, cDNA fragments were generated by RT-PCR of normal spleen total RNA28. Primer sequences and locations are listed in Table 1.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

For the preparation of red cell ghost proteins, whole blood was collected in acid citrate dextrose by retro-orbital phlebotomy. Hemoglobin-depleted ghosts were prepared as previously described[2] by lysis at 4°C in 5 mM $NaPO_4$, pH 7.6, with or without 2 mM $MgCl_2$, containing 1 mM EDTA, 1 mM Pefabloc, 100 μM leupeptin, and 1 μM pepstatin A. After extensive washing in lysis buffer, red cell ghost preparations were analyzed by SDS-PAGE using a 4% Steck gel followed by staining with Coomassie blue33. For western analysis, samples were separated by SDS-PAGE using 10% or 12.5% Laemmli gels[34], transferred to Immobilon-P (Millipore Corporation, Billerica, MA), and probed using the antibodies listed below. Protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

For preparation of tissue homogenates[2], mice were first anesthetized with tribromoethanol, then perfused with PBS to remove RBCs. Tissues were removed, snap frozen in liquid nitrogen, and stored at −80°C until further use. Samples were then homogenized on ice in 0.32 M sucrose containing 2 mM EDTA, 1 mM Pefabloc, 1 μM leupeptin, and 1 μM pepstatin A. Nuclei and debris were removed by centrifugation at 1000 × g for 5 minutes at 4°C. Samples were then fractionated into pellet (membrane) and supernatant (cytosol) fractions by centrifugation at 30,000 × g for 30 minutes at 4°C. The supernatant fraction was removed and the pellet fraction was resuspended in 20% SDS; both pellet and supernatant fractions were made 1x with Laemmli buffer[34], boiled for 5 minutes, and used immediately or stored at −80C until use. Samples were separated using a 10% or 12.5% Laemmli gel34 followed by staining with Coomassie blue or by western analysis.

The following antibodies were used: α-adducin (sc-25731), β-adducin (sc-25732), γ-adducin (sc-25733), tropomyosin (CH1) (sc-58868) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); tropomodulin (LS-C32433) from LifeSpan Biosciences (Seattle, WA); CapZα (mAb 5B12.3) and Cap Zβ (mAb 3F2.3), from the Developmental Studies Hybridoma Bank, The University of Iowa; β-actin (ab8227) and GAPDH (ab9485), from Abcam Inc (Cambridge, MA). Protein 4.9 (dematin), protein 4.1, and glycophorin C antibodies were provided by Dr. Mohandas Narla (New York Blood Center, NY, NY); p55 antibody by Dr. A. H. Chishti (University of Illinois College of Medicine, Chicago, IL). Secondary antibodies were purchased from Abcam or BioRad (Hercules, CA).

Western blots were scanned and densitometry was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/). The average relative changes in adducin amounts were determined by scanning films from two (kidney, spleen, platelets) or three (brain) independent experiments.

Red Blood Cell Analysis

Whole blood was collected by retro-orbital phlebotomy anticoagulated with EDTA, and analyzed using an automated hematology analyzer (ADVIA 120 Multispecies Hematology Analyzer, Bayer Diagnostics, Tarrytown, NY). Analysis conditions were optimized for mouse whole blood. Osmotic fragility of RBCs was measured as previously described[25,35] using three mice of each genotype, each measured in duplicate. Ektacytometry was performed as previously described36 using four mice of each genotype.

Platelet Analysis

Platelet proteins were prepared and analyzed by western blotting as previously described[25]. Bleeding times were done by tail-tip method[37].

Electron Microscopy

Whole blood was collected in acid citrate dextrose by retro-orbital phlebotomy. Red blood cells were washed twice with PBS, fixed in 2% gluteraldehyde in cacodylate buffer (pH 7.4, 4°C), and analyzed using a Hitachi 3000N VP (Hitachi High Technologies America, Inc., Pleasanton, CA) scanning electron microscope, as previously described[2]. Transmission electron microscopy of blood platelets was performed as previously described38.

Measurement of Blood Pressure

Blood pressure and heart rate were determined using a non-invasive tail cuff and pulse transducer system (BP2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC, USA), as described previously[39].

Statistical Analysis

A two-tailed Student's *t* test was used to identify significant differences between mean values.

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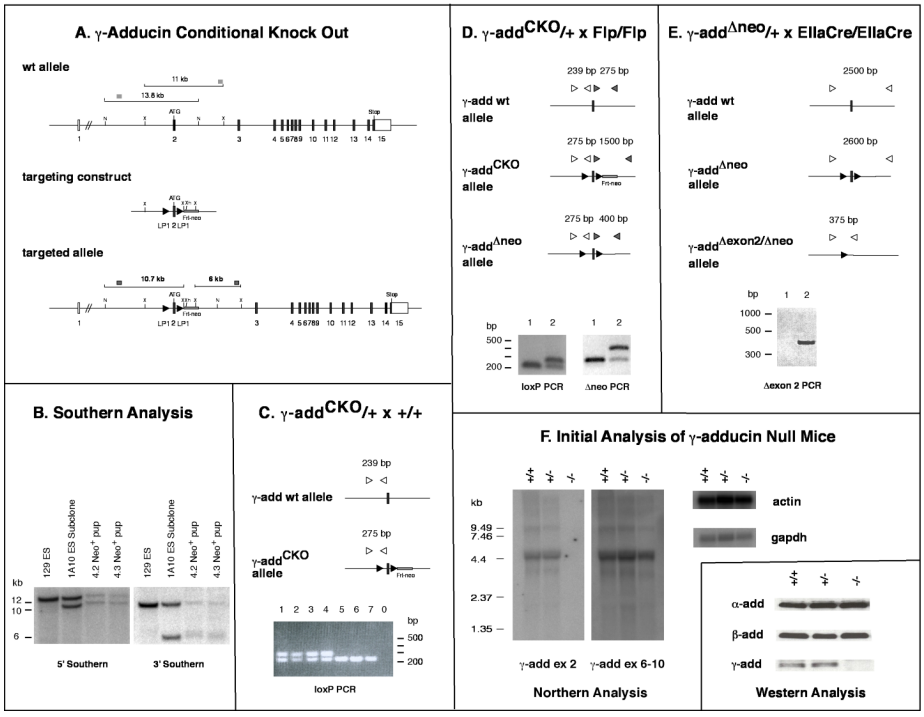

Fig. 1.

Targeted disruption of the γ-adducin gene. (A) A targeting construct was made by inserting loxP sites (LP1 and LP2) upstream and downstream of the first protein coding exon (exon 2), and a Neo^r gene flanked by FRT sequences (Frt-neo) into the downstream intron. The following restriction enzyme sites are indicated: N, *Nde*I; Xh, *Xho*I; X, *Xba*I. Sizes of the predicted 5′ and 3′ restriction fragments and locations of the 5′ and 3′ probes (shaded boxes) are indicated. (B) A correctly targeted G418^r ES cell clone (1A10) was identified by Southern analysis using 5′ and 3′ probes and used for blastocyst injection. Correctly targeted mice (γ-add^{CKO}/+; littermates 4.2 and 4.3) were obtained by mating a high chimera male with a C57BL/6J female were identified by PCR of the neo^r gene and confirmed by Southern analysis. (C) PCR analysis of a litter generated by mating a γ-add^{CKO}/+ male and wt female mouse is shown. The PCR primers used flank the upstream loxP site and generate a 239 bp and 275 bp product from wt and targeted DNA, respectively. (D) The neo^r gene was excised by mating a correctly targeted mouse to a homozygous *Flp* mouse. As indicated, PCR primers flanking the Neo^r gene insertion site generate 275 bp, 1500 bp, and 400 by products from wt, γ-add^{CKO}, and γ-add$^{\Delta neo}$ DNA, respectively. (E) A null mouse lacking exon 1 was then generated by mating a γ-add$^{\Delta neo}$/+ female with an EIIa-cre/EIIa-cre male mouse. Littermates with an excised exon 1 are identified by a 375 bp product. Representative PCR products of +/+ and heterozygous targeted DNA are shown in (D) and (E). (F) Total RNA isolated from the spleens of +/+, +/−, and −/− littermates was analyzed by northern hybridization using cDNA fragments corresponding to γ-adducin exon 2, γ-adducin exons 6-10, actin, and gapdh. 20 μg of total RNA was used for the γ-adducin analysis, while 2 μg of total RNA was used for actin and gapdh analysis. The absence of γ-adducin protein in null mice was verified by western analysis of spleen tissue homogenates (20 μg protein per lane) from littermates generated by a +/− intercross.

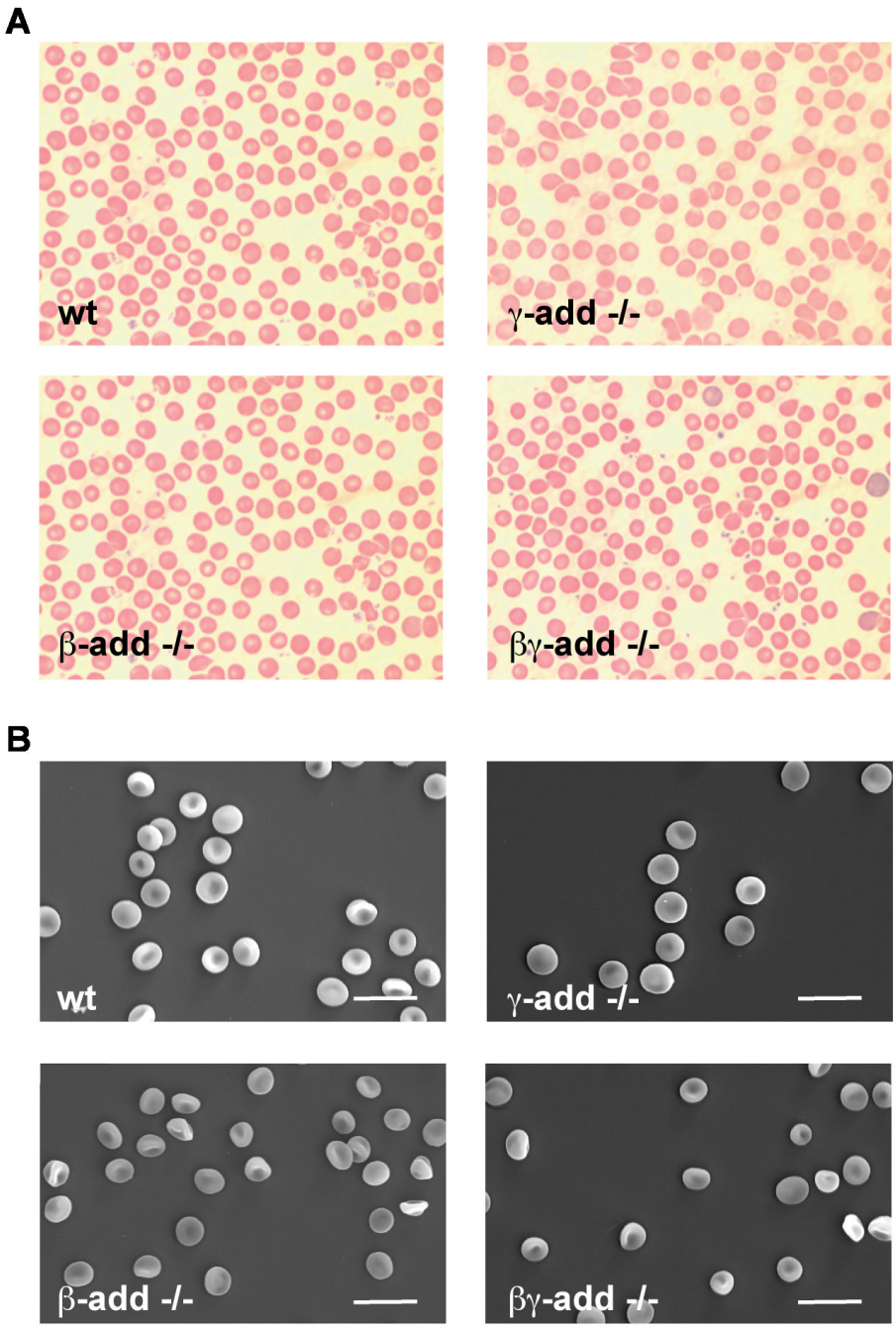

Fig. 2.
Red blood cell morphology. (A) Bright field light microscopy (original magnification 1000x) and (B) scanning electron microscopy of red blood cells isolated from wt, β-adducin null, γ-adducin null, and β/γ-adducin null mice. Scale bar is 10 μM.

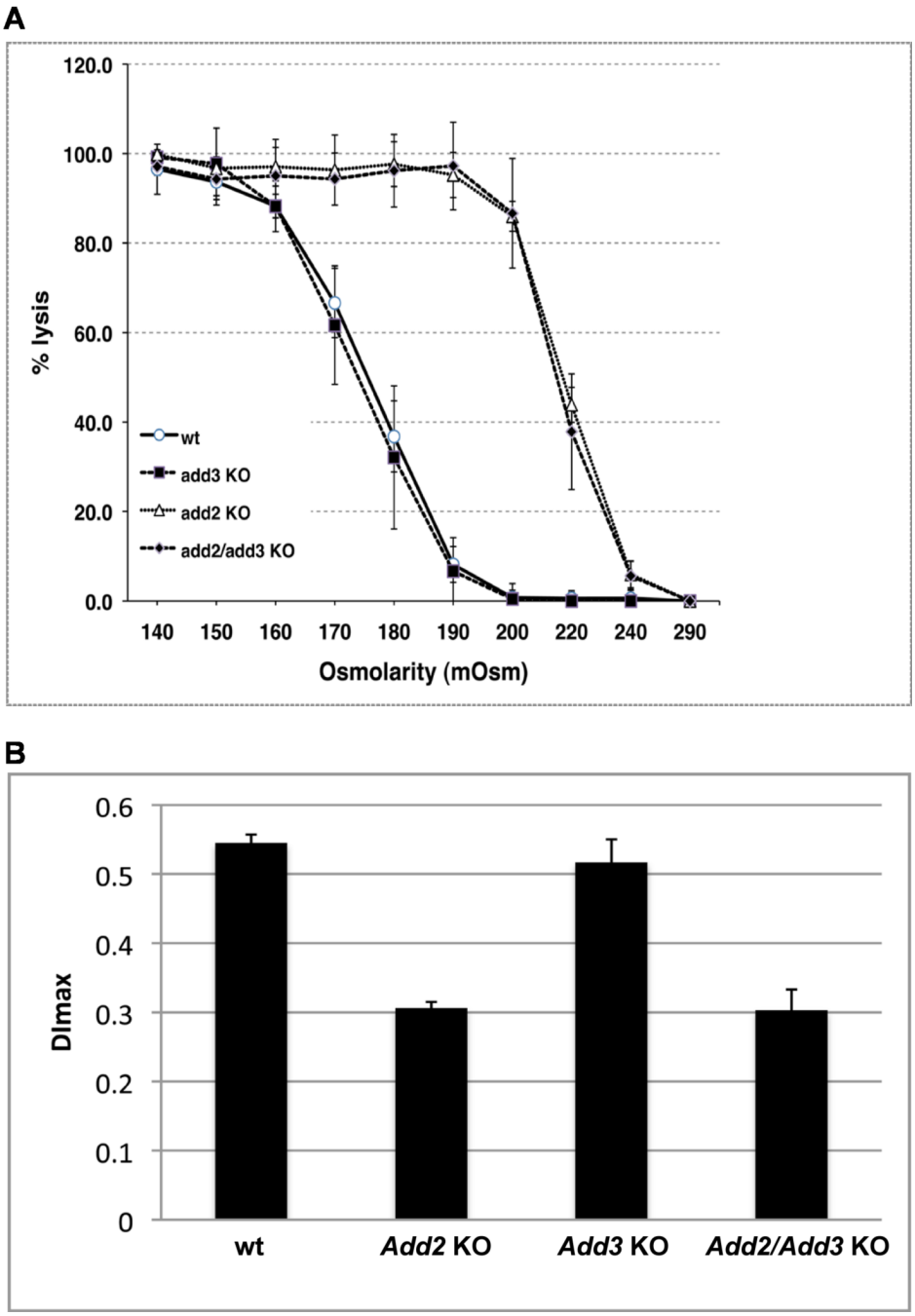

Fig. 3.

Analysis of red blood cells by osmotic fragility and ektacytometry. A. Osmotic fragility was measured using whole blood collected from wt, β-adducin null, γ-adducin null, and β/γ-adducin null mice. Three mice of each genotype were used, each measured in duplicate. B. Ektacytometry was performed using four mice per genotype. By either assay, RBCs isolated from wt and γ-adducin null mice have almost identical profiles, while RBCs isolated from β-adducin null and γ/β-adducin null mice display equally increased fragility.

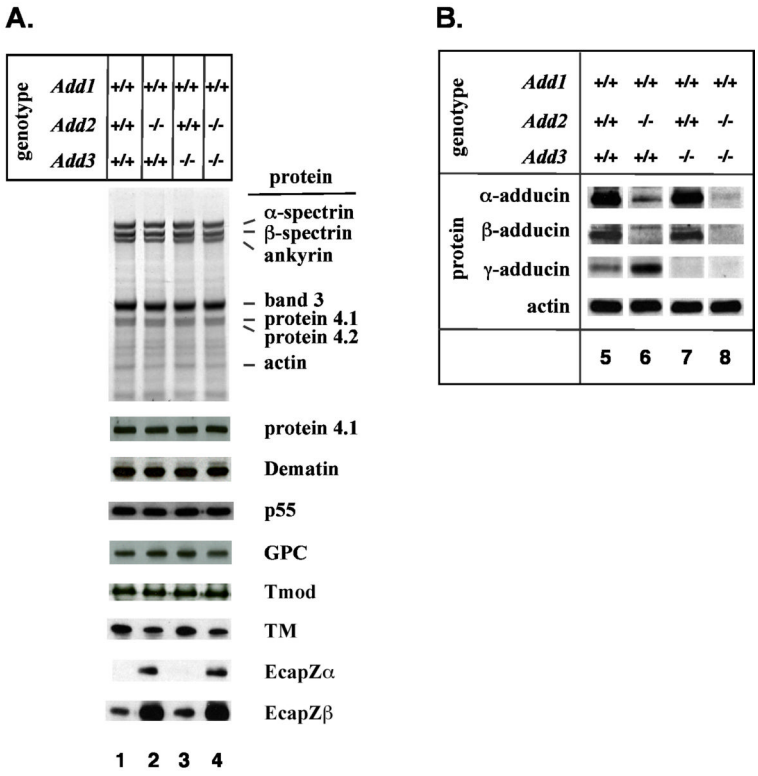

Fig. 4.

Western analysis of red blood cell ghost proteins. (A) Red blood cell ghost proteins were prepared from wt, β-adducin null, γ-adducin null, and γ/β-adducin null mice. The loss of γ-adducin alone (lane 3) has no significant impact on the relative amounts of several membrane skeleton proteins, including spectrin, ankyrin, band 3, proteins 4.1, 4.2, and 4.9 (dematin), p55, glycophorin C (GPC), tropomodulin (Tmod), α-tropomyosin (TM), E-capZα, and E-capZβ. While the loss of β-adducin affects the relative amounts of several of these proteins (lane 2), including actin, α-tropomyosin, E-capZα, and E-capZβ the additional loss of γ-adducin (lane 4) causes no further change in the relative amounts of these proteins. Tmod, TM, E-capZα, and E-capZβ protein amounts were measured using ghosts prepared in the presence of 2 mM $MgCl_2$. As shown in (B), the loss of γ-adducin alone in RBCs has no significant effect on the relative amounts of α- or β-adducin (lane 7). As previously described[2], in the absence of β-adducin (lane 6) the relative amount of α-adducin is significantly decreased while the level of γ-adducin is increased. The loss of both β- and γ-adducin (lane 8) has a dramatic effect on the relative amount of α-adducin, reducing it to an almost undetectable level. (All lanes for each protein are from a single gel but rearranged). Representative results from two independent experiments are shown.

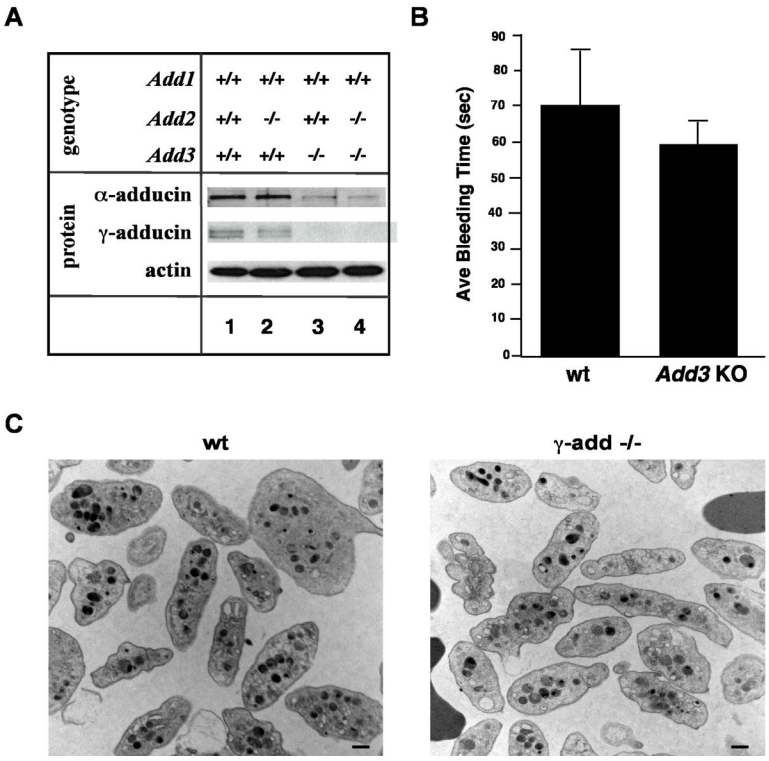

Fig 5.

Analysis of blood platelets. A. The loss of γ-adducin in platelets (lanes 3 and 4) results in a significant decrease (~70%) in the amount of α-adducin. (β-adducin was not detected in platelets.) A representative result of two independent experiments is shown. B. Bleeding times (mean ± standard error) were compared using five wt and six γ-adducin KO age-matched mice. No significant difference was detected in the two groups. C. TEM of wt and γ-adducin KO platelets. Scale bar is 500 nm.

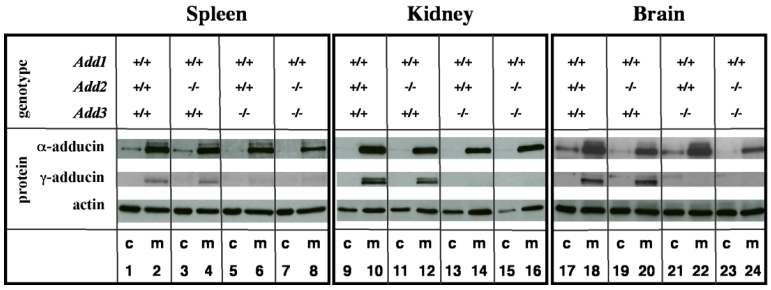

Fig. 6.

Western analysis of nonerythroid cells. Crude cytosol (c) and membrane (m) fractions were prepared from spleen, kidney, and brain tissues of wt, β-adducin null, γ-adducin null, and β/γ-adducin double null mice. The combined loss of β- and γ-adducin decreased α-adducin in the spleen (lanes 7, 8) and brain (lanes 23, 24) by ~50%, and by ~15% in the kidney (lanes 15 and 16). A representative result of two (kidney and spleen) or three (brain) independent experiments is shown.

Table 1

RT-PCR hybridization probes

Gene	Ensemble transcript ID	Exon(s)	Size (bp)	Forward Primer	Reverse Primer
γ-adducin	ENSMUST00000111741	2	216	agaggaagacgactggaga	cagggctctgcaggatctga
γ-adducin	ENSMUST00000111741	6-10	638	aggggagcactgatctgaaa	gaaggcagtcacggttgc
Actin	ENSMUST00000031564	3-6	929	gaggcccagagcaagag	ccggactcatcgtactc
Gapdh	OTTMUSG00000027118	3-5	638	caacgacccttcattgacctc	atccacgacggacacattgg

Table 2

Hematological Values

	wt (n=10)	γ-add −/− (n=10)	β-add −/− (n=5)	β-add/γ-add −/− (n=6)
RBC (10^6 cells/μl)	10.20 ± 0.25	10.34 ± 0.25	10.33 ± 0.43	10.38 ± 0.27
HGB (g/dL)	15.97 ± 0.39	15.92 ± 0.37	15.52 ± 0.74	15.38 ± 0.32
HCT (%)	48.25 ± 1.58	48.44 ± 0.75	45.46 ± 2.32*	45.45 ± 0.97*
MCV (fL)	47.32 ± 1.13	46.87 ± 0.93	43.98 ± 1.22*	43.83 ± 0.88*
MCH (pg)	15.67 ± 0.34	15.38 ± 0.34	15.02 ± 0.24*	14.83 ± 0.14*
MCHC (g/dL)	33.11 ± 0.85	32.86 ± 0.50	34.14 ± 0.66*	33.83 ± 0.41^
CHCM (g/dL)	31.29 ± 0.76	31.27 ± 0.43	32.54 ± 0.88*	32.35 ± 0.52*
RDW (%)	13.31 ± 0.58	12.95 ± 0.94	14.32 ± 0.41*	14.82 ± 0.58*
HDW (g/dL)	1.91 ± 0.09	1.93 ± 0.07	2.38 ± 0.19*	2.41 ± 0.11*
Retic (%)	3.41 ± 0.58	3.34 ± 0.41	5.42 ± 0.90*	5.78 ± 1.14*
PLT (10^3 cells/μl)	1148 ± 297	1189 ± 82	1259 ± 246	1319 ± 147
MPV (fL)	5.57 ± 0.46	5.68 ± 0.35	5.54 ± 0.26	5.38 ± 0.18

All mice 6-8 weeks of age

All values mean ± standard deviation

RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin content; CHCM, corpuscular hemoglobin concentration mean; RDW, red cell distribution width; HDW, hemoglobin distribution width; Retic, reticulocytes; PLT, platelet count; MPV, mean platelet volume

* $p<0.05$ compared to wt

^ $p<0.075$ compared to wt

Table 3

Blood Pressure Values

	wt (n=10)	γ-add −/− (n=9)
Systolic Pressure	127.69 ± 20.80	128.31 ± 23.96
Mean Arterial Pressure	98.19 ± 23.96	100.18 ± 24.56
Diastolic Pressure	83.44 ± 27.92	86.12 ± 29.07
Pulse Rate	595.38 ± 52.88	596.25 ± 44.01

All mice were female and age matched.

All values mean ± standard deviation.