Published in final edited form as:
Genesis. 2010 September ; 48(9): 531–539. doi:10.1002/dvg.20657.

Appl1 is Dispensable for Akt Signaling *in vivo* and Mouse T Cell Development

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Abstract

Appl1 (Adaptor protein containing pleckstrin homology [PH], phosphotyrosine binding [PTB], and Leucine zipper motifs) is an adaptor that participates in cell signaling by interacting with various signaling molecules including Akt, PI3-kinase (PI3K), Rab5, adiponectin receptor and TrkA. By using RNA knockdown technology, Appl1 has been implicated in zebrafish development and murine glucose metabolism. To investigate the unambiguous role of Appl1 *in vivo*, we generated a knockout mouse in which exon1 of the *Appl1* gene was disrupted using gene trap methodology. However, homozygous *Appl1* knockout mice with ubiquitous loss of Appl1 protein expression were viable, grossly normal, and born at expected Mendelian ratios. Moreover, activation of Akt and the downstream effecter Gsk3β were unaffected *in vivo*. We next performed glucose and insulin tolerance tests and found the glucose metabolism is normal in *Appl1*-null mice. We also tested the effect of Appl1 loss on Akt signaling in T cells, because we have discovered that Appl1 strongly interacts with the p110β subunit of PI3K in T lymphocytes. However, such interaction was found to be dispensable for Akt signaling in thymic T cells and T cell development. Moreover, Appl1 loss did not affect DNA synthesis in cultured thymocytes, although loss of Appl1 was associated with a slight increase in ConA-stimulated splenic T cell viability/proliferation. Collectively, our findings indicate that Appl1 is dispensable for Akt signaling *in vivo* and T cell differentiation.

Keywords

PI3 kinase; Akt signaling; T cell development; Glucose metabolism; knockout mouse model

Introduction

Appl proteins are membrane-bound adaptors that interact with many signaling molecules involved in a variety of signal transduction pathways. Appl proteins contain an N terminal Bar domain, a PH domain, and a C-terminal PTB domain (Habermann, 2004). Appl1 was first discovered as a binding partner of Akt and the p110 subunit of PI3-kinase (PI3K) (Mitsuuchi *et al*., 1999). Subsequently, Appl1 was found to bind to TrkA in neuronal cells to promote nerve growth factor signaling to ERK and Akt (Varsano *et al*., 2006). Also, Appl1 binds to the adiponectin receptor and facilitates signaling to p38 MAPK and AMPK (Mao *et al*., 2006). Moreover, Appl is required for the small GTPase Rab5 to transduce cellular signals from the endosome to the nucleus (Miaczynska *et al*., 2004). In addition to its role in

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mitogen signaling, Appl has been implicated in apoptotic signaling. Knockdown of Appl inhibits cell cycle progression in HeLa cells and has been reported to cause embryonic lethality in zebrafish by promoting apoptosis (Miaczynska *et al*., 2004; Schenck *et al*., 2008). Moreover, APPL interacts with the tumor suppressor DCC (deleted in colorectal cancer), which is required for the latter's apoptotic function (Liu *et al*., 2002). Interestingly, knockdown of Appl1 by shRNA has been shown to attenuate insulin-stimulated glucose metabolism by inhibiting Akt activity in mice (Cheng *et al*., 2009).

We have recently generated a knockout model in which exon5 of the *Appl1* gene was flanked by *LoxP* sites, and deletion of *Appl1* by crossing with EIIA-Cre mice abolished Appl1 protein expression in all tissues tested. Unexpectedly, *Appl1* deficiency rendered no obvious phenotypic abnormalities, and these *Appl1*-null mice have grossly normal Akt signaling *in vivo* (Tan *et al*.). To validate this knockout mouse model, we have developed and characterized a second *Appl1*-null mouse model, using a different knockout strategy. Here, we report that disruption of exon1 of the *Appl1* gene by a gene trap approach produces no gene product. As with our earlier model, Appl1 was found to be dispensable for embryonic development and postnatal growth. We also show that Appl1 is highly associated with the p110β subunit of Pi3k in thymic T cells, although this association does not appear to affect Akt activation or T cell differentiation. Moreover, Appl1 appeared to be dispensable in insulin-triggered glucose metabolism.

Results

Appl1 is not required for mouse development

In this *Appl1* knockout mouse model, exon1 of the *Appl1* gene is disrupted by gene trap insertion, which interrupts *Appl1* transcription and results in no detectable mRNA in homozygous knockout mice (Fig. 1a-c). Like the *Appl1* conditional knockout mouse model in which exon5 is deleted, the homozygous knockout mice reported here are viable and have grossly normal development. In addition, *Appl1*-null mice from heterozygous x heterozygous crosses were born at the predicted Mendelian ratio (Table 1).

Appl1 is dispensable for Akt stability and activation *in vivo*

Like the *Appl1* exon5 knockout, the gene trap insertion into exon1 of *Appl1* ablated expression of the gene as shown by western blot analysis of various adult mouse tissues (Fig. 2a). Notably, there was compensating up-regulation of Appl2 protein only in lung tissue. Moreover, Appl1 loss did not affect the stability of Akt family proteins. To rule out the potential interference of variable circadian rhythms among individual mice (Ko *et al*., 2009), the levels of phosphorylated Akt in fetal brain and lung from multiple *Appl1*−/−, *Appl*1+/−, and wild-type littermates were compared. No evidence of compromised level of phospho-Akt, phospho-Gsk3 or phospho-Tsc2 was observed in the tissues tested, suggesting that Appl1 is dispensable for Akt activation *in vivo* (Fig. 2b-c).

Appl1 is dispensable for insulin-triggered glucose metabolism

To validate previous work demonstrating that *Appl1* knockdown by an adenoviral shRNA impairs insulin action and induces hyperglycemia (Cheng *et al*., 2009), we measured glucose metabolism under normal, glucose challenge or insulin stimulation conditions. When eating *ad lib*, *Appl1*-null mice had normal blood glucose levels (Suppl. Fig. 1a). After fasting for 16 h, a glucose tolerance test was performed, which revealed that *Appl1*-null mice exhibit normal glucose clearance ability (Suppl. Fig. 1b). We next performed an insulin tolerance test in which mice were fasted for 6 h, and then insulin solution was injected at 0.75 U/kg body weight. This experiment revealed that *Appl1*-null mice do not have a defect in insulin response (Suppl. Fig. 1c).

Appl1 is highly bound to Pi3k p110β in thymic T cells

We previously showed that APPL1 binds to the p110α subunit of PI3K (Mitsuuchi *et al*., 1999). To identify tissues that might be especially susceptible to Appl1 loss, IP/western blot analysis was performed on various mouse tissues. With the exception of kidney, expression of Appl1 protein was abundant in most tissues. Pi3k p110α and p85 were expressed at similar levels in most mouse tissues, whereas p110β was highly expressed in organs involved in immunity and reproduction (Fig. 3a). P110γ, on the other hand, was highly expressed in thymic T cell and spleen. In co-IP experiments on various mouse tissues, Appl1 was found to strongly interact with p110β in thymic T cells compared with its interaction in other tissues. It is noteworthy that the binding pattern of Appl2 was very similar to that of Appl1, implying a possible redundant role between these two Appl proteins (Fig. 3b). The amount of Appl1/2-associated p110β in thymic T cells was measured by comparison with 5% input of p110β IP (Fig. 3c). The relative amount of association in other tissues was also compared (Fig. 3d). The amount of Appl-associated p110α or P110γ appeared to be small (Fig. 3c, e). A reciprocal co-IP was performed to verify Appl1-p110β binding (Fig. 3e). The specificity of the Appl1-p110β interaction was further demonstrated by co-IP experiments on brain tissues from wild-type and *Appl1*-null mice (Fig. 3f).

Appl1 is dispensable for Akt activation in thymic T cells

To evaluate the significance of the interaction between Appl1 and Pi3k p110 in thymic T cells, levels of phosphorylated Akt were compared among thymic T cells from wild-type and *Appl1*-deficient littermates at postnatal day 2. Surprisingly, Akt activation was not affected by Appl1 loss, suggesting a possible compensatory effect by Appl2 or that such an interaction is dispensable for the function of Pi3k and Akt in the thymus (Fig. 4).

Appl1 loss does not affect peripheral T cell distribution

Lymphocyte numbers were unaltered in Appl1 knockout mice in which exon5 of the *Appl1* gene is deleted (Tan *et al*.). To determine if Appl1 loss alters lymphoid progenitor cell commitment into T cells, peripheral lymphocytes were analyzed for distribution of T and B cell populations. No difference in T and B cell differentiation was observed as shown by the CD3+/CD19+ ratio (Fig. 5a). To determine if Appl1 is required for early T cell fate determination through Akt independent pathways, the TCR α/β and γ/δ positive populations were measured by FACS. The ratio of TCR α/β and γ/δ populations did not differ between wild-type and *Appl1*-null mice (Fig. 5b). Moreover, the CD4/CD8 T cell ratio was unaltered, suggesting that T cell development is intact in *Appl1*-null mice (Fig. 5c). Likewise, we did not see differences in the ratios of TCR α/β and γ/δ populations or in the ratio of CD4/CD8 of isolated splenic T cells (Suppl. Fig. 2).

Appl1-deficiency does not affect T cell development in the thymus

Gross analysis indicated that neither the overall thymic cellularity, nor the major subsets of thymocytes were significantly altered: CD4-CD8- (double-negative; DN), CD4+CD8+ (double-positive; DP) or CD4+ or CD8+ single positive (SP). The lone exception was that the number of CD4 SP cells was slightly elevated (Fig. 6a). Detailed analysis of the major checkpoints of T cell development, which are dependent upon T cell receptor (TCR) signaling (β-selection, αβ/γδ lineage commitment, and positive selection), likewise revealed no major defects in development. The pre-TCR complex is triggered by productive rearrangement of the TCRβ locus at the CD44-CD25+ DN3 stage, resulting in development to the CD44-CD25- DN4 stage (von Boehmer and Fehling, 1997) (Fig. 6b). Both CD5 induction and the numbers of DN3 and DN4 cells in *Appl1*-deficient mice are indistinguishable from those observed in wild type mice, suggesting that β-selection occurs normally (Fig. 6c, d). Neither total numbers of γδ lineage thymocytes nor their maturation to

the HSA^{lo} phenotype was altered by *Appl1*-deficiency, indicating that Appl1 is not essential for the TCR signals driving αβ/γδ lineage commitment (Fig. 6e). This interpretation is further supported by the absence of changes in frequency or number of Vγ2 and Vγ1.1 thymocytes, which are major γδ lineage subsets whose development is altered by perturbations in TCR signaling (Lauritsen *et al*., 2009) (Fig. 6f). Finally, positive selection and CD4/8 lineage commitment also appeared to occur normally in *Appl1*-deficient mice (He and Kappes, 2006) (Fig. 6g). The number of positively selected cells that are $TCR\beta^{hi}CD69^{hi}$ was not altered. Likewise, while the number of $TCR\beta^{hi}CD69^{lo}$ mature cells was increased in *Appl1*-deficient mice, the increase was quite modest (Fig. 6h). Taken together, these data demonstrate that Appl1 does not play an essential role in regulating the TCR-dependent phases of T cell development in the thymus.

Loss of Appl1 has minimal effects on T cell proliferation

To analyze short-term thymocyte proliferation, the number of primary thymocytes synthesizing DNA was assessed by pulse-labeling with BrdU. No difference was observed between the two genotypes (Fig. 7a and Suppl. Fig. 3). To test the viability/proliferation rate of mature T cells following ConA stimulation, purified spleen T cells were incubated with ConA-conditioned medium for 4 days. Interestingly, the MTS assay showed a slightly higher proliferative activity in T cells from *Appl1*-deficient mice compared to that seen in wild-type littermates (Fig. 7b). Similar findings were observed in splenic T cells from Appl1 exon5 knockout mice (data not shown).

Discussion

Appl1 has been reported to be an essential adaptor protein involved in signaling initiated from adiponectin, NGF, and insulin receptors which lead to the activation of various signal transduction pathways including the AKT, ERK, p38 MAPK and AMPK pathways *in vitro* (Mao *et al*., 2006; Saito *et al*., 2007; Varsano *et al*., 2006). However, the ubiquitous Appl1 knockout mouse that has exon5 excised through Cre-mediated recombination develops normally with no obvious defect in Akt signaling in various tissues tested (Tan *et al*.). Here we report a second *Appl1*-null mouse model in which exon1 is disrupted and thereby results in no gene product. Mice from this knockout model are also grossly normal, with unaltered Akt signaling in various tissues. These two Appl1 models unambiguously support the notion that Appl1 is dispensable for mouse embryonic organogenesis and postnatal growth.

It was recently reported that *Appl1* knockdown by 40-80% in mice causes insulin insensitivity and hyperglycemia (Cheng *et al*., 2009). However, our Appl1 knockout mice demonstrated normal responses in glucose tolerance and insulin tolerance tests. Given that no rescue experiment with a wobbled Appl1 cDNA was performed (Clark *et al*., 2008; Jackson *et al*., 2003; Persengiev *et al*., 2004; Scacheri *et al*., 2004), it is possible that the reported defect was due to an off-target effect of the Appl1 silencing by shRNA. However, we also cannot rule out an acute effect caused by the diminished levels of Appl1 protein.

To identify possible tissue-specific effects of Appl1 loss, we performed co-IP experiments. Interestingly, the amount of Appl-associated Pi3k p110β was found to be extremely high in thymic T cells, although the protein levels of Appl1 and Pi3k p110β in thymus do not vary much from that observed in other tissues.

The expression of p110α and p110β are uniform among tissues compared with that of p110γ. Accordingly, knockout of the p110α or p110β genes causes embryonic lethality, which implies that the function of each of these Pi3k subunits is required ubiquitously and is not redundant (Bi *et al*., 2002; Bi *et al*., 1999). T cell specific knockout of the p110α gene, *Pik3ca*, in mice leads to autoimmunity in the lacrimal glands, although no effect on T cell

development was reported (Oak *et al*., 2006). The effect of p110β loss on T cell development has not been reported to date. We found that Pi3k p110γ is highly expressed in thymic T cells as well as in spleen, which is consistent with the fact that p110γ knockout mice have defects in T cell differentiation (Sasaki *et al*., 2000). A kinase-inactivating mutation in the p110δ isoform of Pi3k results in normal T cell development, but impaired TCR-stimulated cell proliferation *in vitro* (Okkenhaug *et al*., 2002). Interestingly, p85α knockout mice have a profound defect in B cell maturation due to the lack of Pi3k activity specifically in B cells, not T cells (Fruman *et al*., 1999). This was thought to be compensated by p85β, because the PI3K inhibitor LY294002 inhibits TCR-dependent proliferation in p85α-null T cells (Okkenhaug and Vanhaesebroeck, 2003). Together, during T cell development, p110γ and p110δ have an indispensable role, but the role of p110α may be negligible, and p110β's role is still not clear.

Our study suggests that the Appl1 is dispensable for Akt activation *in vivo* and thereby does not seem to affect T cell differentiation or glucose metabolism. However, we did find that *Appl1*-null splenic T cells have a slightly increased proliferative response to ConA, suggesting that Appl1 is required for homeostasis under certain stress conditions. Moreover, because of a possible redundant role between Appl1 and Appl2, *Appl1/2* double knockout mice would provide a better model to investigate the *in vivo* function of Appl in future studies.

Material and Methods

Generation of Appl1 knockout mice

ES cells harboring a gene trap inserted in *Appl1* exon1 (clone xmo73) were purchased from Bay Genomics (San Francisco, CA). In brief, a *β-geo* cassette from vector pGT0lxf was randomly inserted in *Appl1* exon 1 through a gene trap strategy, and the specific genomic location was identified by sequencing (Fig. 1a). Chimeric mice were generated by injecting ES cells into C57BL/6 blastocysts. The chimeras were back-crossed with C57BL/6 mice at least eight times before initiating the experiments. Primers used for genotyping were: GAT CGA CAA GCT GCC CAT TG (primer1 in exon1), GAA CAG GAC TTA TCT CAC ATC C (primer2 in intron1) and CAT CCA CTA CTC AGT GCA GTG (primer3 in pGT0lxf). The PCR product for the wild-type allele is 350 bp and the targeted allele is 568 bp (Fig. 1b). Primers used to detect 5′ and 3′ cDNA sequences were as follows: For N' cDNA PCR: forward – CAT TGA AGA GAC CCT GGA GG (within exon1) and reverse – ACT GGG AAA TGG GGA ACA TC (within exon5). For C' cDNA PCR: forward – AGA TCT TAG CTG CTC GGG C, reverse – TGG TTT GGT CTA CTG GAG GC. The following conditions were used for PCR: denaturation at 94° C for 3 min, followed by denaturation at 94° C for 20 sec, annealing at 57° C for 30 sec, and extension at 72° C for 50 sec for 30 cycles. Animal work was conducted according to the protocol of the Institutional Animal Care and Use Committee of the Fox Chase Cancer Center.

Glucose tolerance and insulin tolerance assays

Glucose tolerance and insulin tolerance assays were performed as described (Cho *et al*., 2001). For the glucose tolerance test, mice were starved for 16 h before I.P. injection of glucose (75mg/ml glucose solution, 1.5 mg glucose/g body weight), with blood glucose levels being monitored at 0, 20, 40, 60 and 120 min by using a glucometer (Bayer Healthcare LLC, Mishawaka, IN). For the insulin tolerance test, mice were starved for 6 h before I.P. injection of human recombinant insulin (0.75 U/kg body weight), and glucose levels were monitored at 0, 20, 40, 60 and 120 min.

Isolation of thymic and peripheral lymphocytes

Thymi from 2-3-month-old mice were collected and washed twice with cold phosphate buffered saline. Thymi were then soaked in cold Iscove's MEM and gently passed through a 100-μm mesh (BD Biosciences, Bedford, MA). The T cell suspension was spun down at 1,200 rpm for 10 min. For purification of peripheral T cells, approximately 200 μl of blood was drawn via orbital sinus puncture. Lymphocytes were isolated by using Lympholyte-M solution (Cedarlane Laboratories, Burlington, NC).

Western blot analysis

Total cellular protein was isolated by using cell lysis buffer supplemented with 2 mM PMSF (Cell Signaling Technology, Danvers, MA). Cell debris was removed by centrifuging at 15,000 × g for 15 min at 4°C. Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA). Then 50 μg protein/sample was loaded into Tris-Glycine-buffered SDS-PAGE gel (Invitrogen, Carlsbad, CA). Separated protein was then transferred onto PVDF membranes (Millipore, Billerica, MA). Antibodies against Appl1, PI3K p110α and p110γ, p-Akt/Akt and p-Gsk3/Gsk3 were purchased from Cell Signaling. Antibodies against Appl2, Pi3k p110β, Gapdh and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Density of protein signal was quantified by using AlphaEaseFC (Alpha Innotech Corporation, San Leandro, CA).

Co-Immunoprecipitation

Co-IP was performed by using ExactaCruz IP kits (Santa Cruz Biotechnology). In short, 1 mg protein lysates were pre-cleared with preclearing beads and then incubated with IP matrix-antibody overnight in a cold room on a rotator. Beads were washed three times with lysis buffer, resuspended in 4X loading buffer, and boiled for 5 min before electrophoresis.

Flow cytometric analysis

Fluorescein-conjugated antibodies against TCRβ, TCRγδ (GL3), CD3, CD4, CD5, CD8, CD19, CD 25, CD44, CD69, CD127 and HSA were purchased from BD Pharmingen (San Diego, CA). Cells were stained with specific antibodies at 4°C for 20 min and analyzed by FACScan (BD, San Jose, CA).

Cell proliferation assay

BrdU incorporation assay was performed according to the manufacturer's protocol (BD). In brief, 1×10^6 thymocytes were incubated with 10 μM BrdU for 12 h. Positive cells were stained with FITC-conjugated anti-BrdU antibody and analyzed with FACScan. MTS assay was used to evaluate viability/proliferation of splenic T cells. T cells were purified from single cell suspension of spleen cells by using T-cell enrichment columns (R&D Systems, Minneapolis, MN). Purified cells were cultured without or with stimulation with 5% ConA-conditioned medium (BD) for 4 days. MTS reagent (Promega, Madison, WI) was added at a final concentration of 17%, and then cells were incubated for 4 h before analysis at 490 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The following Fox Chase Cancer Center shared facilities were used in the course of this work: Laboratory Animal, Transgenic Mouse, Flow Cytometry, Cell Culture, and Genomic Facilities. This work is supported by National Cancer Institute Grants CA77429 (J.R.T.) and CA06927 (Fox Chase Cancer Center), and an appropriation from the Commonwealth of Pennsylvania. Y.T. was supported in part by a William J. Avery Postdoctoral Fellowship.

Contact Grant Sponsor: National Institutes of Health; Contact Grants numbers: CA77429 and CA06927

References

Bi L, Okabe I, Bernard DJ, Nussbaum RL. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. Mamm Genome. 2002; 13:169–172. [PubMed: 11919689]

Bi L, Okabe I, Bernard DJ, Wynshaw-Boris A, Nussbaum RL. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. J Biol Chem. 1999; 274:10963–10968. [PubMed: 10196176]

Cheng KK, Iglesias MA, Lam KS, Wang Y, Sweeney G, Zhu W, Vanhoutte PM, Kraegen EW, Xu A. APPL1 potentiates insulin-mediated inhibition of hepatic glucose production and alleviates diabetes via Akt activation in mice. Cell Metab. 2009; 9:417–427. [PubMed: 19416712]

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science. 2001; 292:1728–1731. [PubMed: 11387480]

Clark PR, Pober JS, Kluger MS. Knockdown of TNFR1 by the sense strand of an ICAM-1 siRNA: dissection of an off-target effect. Nucleic Acids Res. 2008; 36:1081–1097. [PubMed: 18096615]

Fruman DA, Snapper SB, Yballe CM, Davidson L, Yu JY, Alt FW, Cantley LC. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science. 1999; 283:393–397. [PubMed: 9888855]

Habermann B. The BAR-domain family of proteins: a case of bending and binding? EMBO Rep. 2004; 5:250–255. [PubMed: 14993925]

He X, Kappes DJ. CD4/CD8 lineage commitment: light at the end of the tunnel? Curr Opin Immunol. 2006; 18:135–142. [PubMed: 16480861]

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol. 2003; 21:635–637. [PubMed: 12754523]

Ko ML, Jian K, Shi L, Ko GY. Phosphatidylinositol 3 kinase-Akt signaling serves as a circadian output in the retina. J Neurochem. 2009; 108:1607–1620. [PubMed: 19166512]

Lauritsen JP, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, Kappes DJ, Zuniga-Pflucker JC, Wiest DL. Marked induction of the helix-loop-helix protein Id3 promotes the gammadelta T cell fate and renders their functional maturation Notch independent. Immunity. 2009; 31:565–575. [PubMed: 19833086]

Liu J, Yao F, Wu R, Morgan M, Thorburn A, Finley RL Jr. Chen YQ. Mediation of the DCC apoptotic signal by DIP13 alpha. J Biol Chem. 2002; 277:26281–26285. [PubMed: 12011067]

Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F, Dong LQ. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol. 2006; 8:516–523. [PubMed: 16622416]

Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, Wilm M, Parton RG, Zerial M. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell. 2004; 116:445–456. [PubMed: 15016378]

Mitsuuchi Y, Johnson SW, Sonoda G, Tanno S, Golemis EA, Testa JR. Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene. 1999; 18:4891–4898. [PubMed: 10490823]

Oak JS, Deane JA, Kharas MG, Luo J, Lane TE, Cantley LC, Fruman DA. Sjogren's syndrome-like disease in mice with T cells lacking class 1A phosphoinositide-3-kinase. Proc Natl Acad Sci U S A. 2006; 103:16882–16887. [PubMed: 17071741]

Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, Pearce W, Meek SE, Salpekar A, Waterfield MD, Smith AJ, Vanhaesebroeck B. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. Science. 2002; 297:1031–1034. [PubMed: 12130661]

Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. Nat Rev Immunol. 2003; 3:317–330. [PubMed: 12669022]

Persengiev SP, Zhu X, Green MR. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA. 2004; 10:12–18. [PubMed: 14681580]

Saito T, Jones CC, Huang S, Czech MP, Pilch PF. The interaction of Akt with APPL1 is required for insulin-stimulated Glut4 translocation. J Biol Chem. 2007; 282:32280–32287. [PubMed: 17848569]

Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A, Penninger JM. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science. 2000; 287:1040–1046. [PubMed: 10669416]

Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc Natl Acad Sci U S A. 2004; 101:1892–1897. [PubMed: 14769924]

Schenck A, Goto-Silva L, Collinet C, Rhinn M, Giner A, Habermann B, Brand M, Zerial M. The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. Cell. 2008; 133:486–497. [PubMed: 18455989]

Tan Y, You H, Wu C, Altomare DA, Testa JR. Appl1 Is Dispensable for Mouse Development, and Loss of Appl1 Has Growth Factor-selective Effects on Akt Signaling in Murine Embryonic Fibroblasts. J Biol Chem. 285:6377–6389. [PubMed: 20040596]

Varsano T, Dong MQ, Niesman I, Gacula H, Lou X, Ma T, Testa JR, Yates JR 3rd, Farquhar MG. GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling. Mol Cell Biol. 2006; 26:8942–8952. [PubMed: 17015470]

von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. Annu Rev Immunol. 1997; 15:433–452. [PubMed: 9143695]

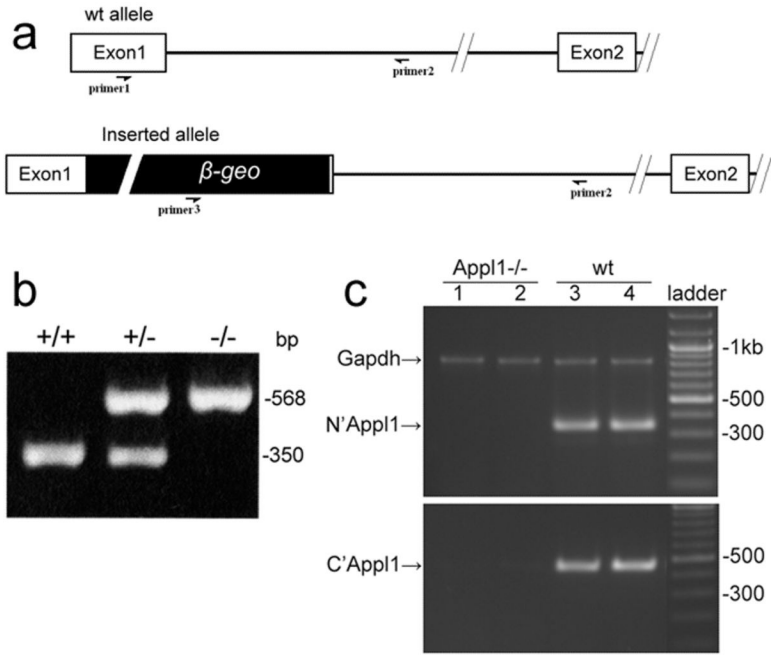

Figure 1.

Targeted disruption of *Appl1* exon1. (a) Diagram illustrating gene trap-based gene disruption. (b) PCR genotyping showing wild-type (wt) and inserted *Appl1* alleles using tail DNA from wt, +/− and −/− mice. (c) Semi-quantitative RT-PCR demonstrating that N' and C' of *Appl1* mRNA is not expressed from homozygous *Appl1* −/− mouse tails.

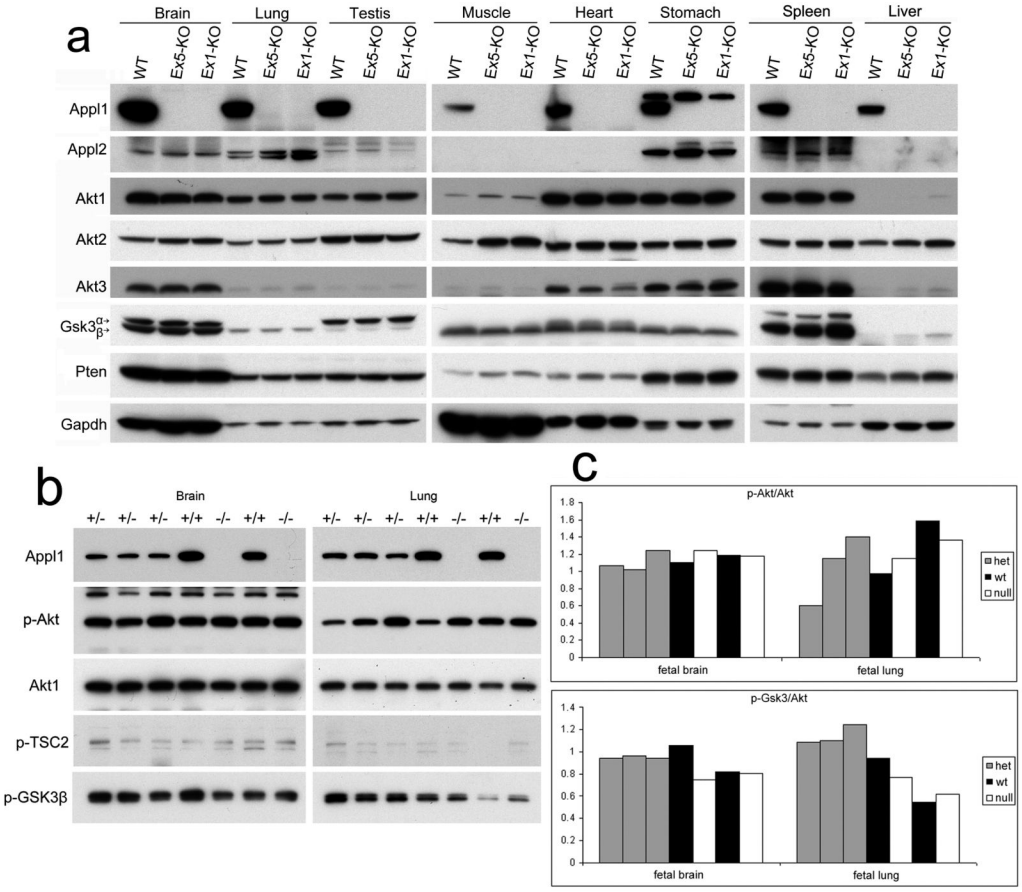

Figure 2.

Loss of Appl1 expression in various adult tissues from exon1 or exon5 *Appl1* knockout mice. (a) Appl1, Appl2, as well as proteins in Akt pathway were analyzed by using immunoblotting. (b-c) Loss of Appl1 does not affect Akt pathway activity in fetal brains and lungs. Embryonic littermates at E18.5 were sacrificed, and brain and lung tissues were collected. P-Akt, Akt and p-Gsk3 levels were analyzed by western blotting and quantified.

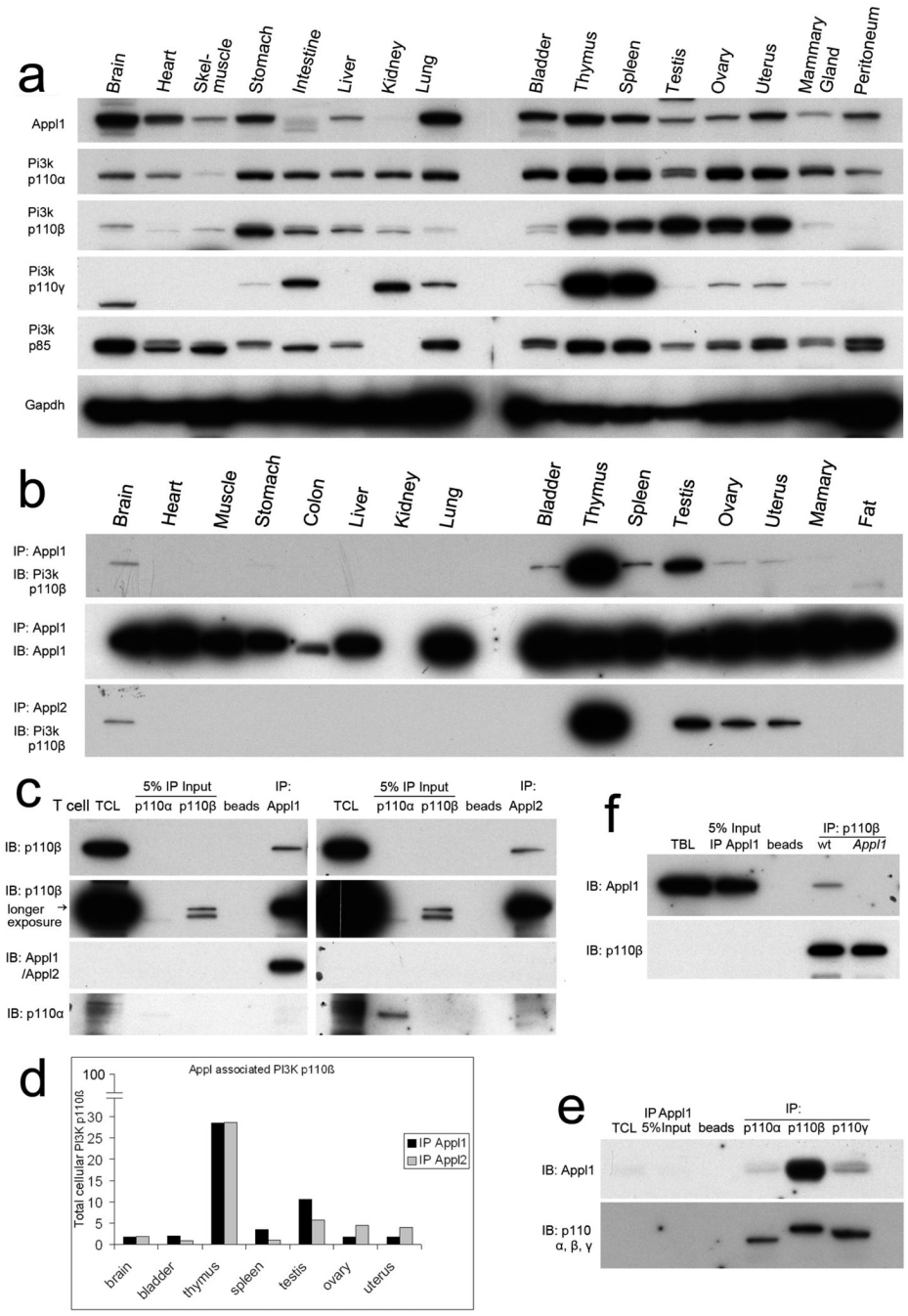

Figure 3.

Thymic T cells possess a high level of Appl-Pi3k p110β interaction. (a) Tissue distribution of Pi3k subunits p110α, p110β, p110γ and p85. (b) co-IP experiments demonstrating that Appl1 and Appl2 interact with the p110β subunit of Pi3k in various mouse tissues. Note that Appl2 antibody only recognizes mouse Appl2 protein in its naïve state. (c) co-IP showing that Appl1 and Appl2 interact robustly with p110β, but not p110α, in thymic T cells. (d) Relative amount of Appl1/2-associated Pi3k p110β subunit among various mouse tissues. (e) Interaction of Appl1 with p110α, p110β and p110γ in thymic T cells. Total T cell lysates were extracted and subjected to co-IP using antibodies against p110α, p110β or p110γ.

Appl1 antibody was used to detect the association. (f) Specificity of Appl1-p110β interaction in wt and *Appl1* −/− brain tissues.

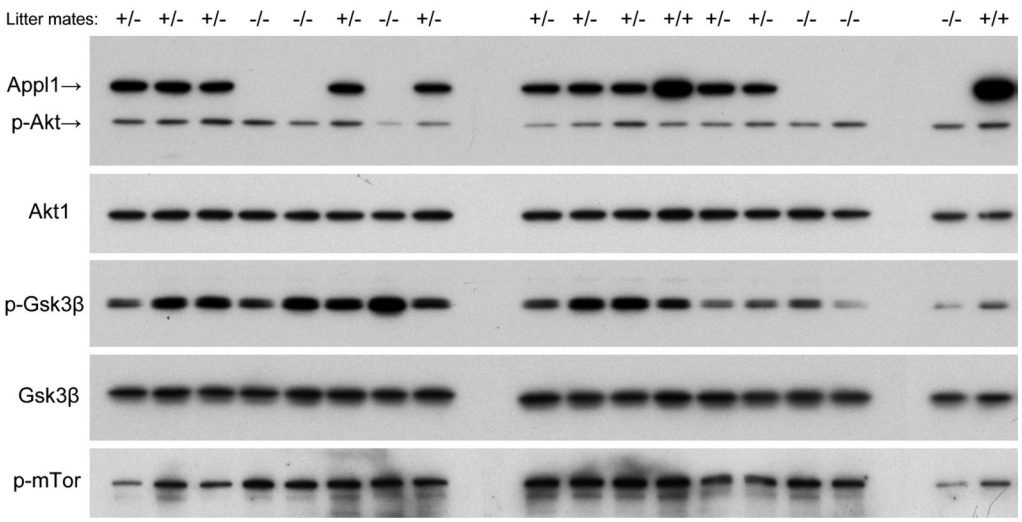

Figure 4.
Appl1 is dispensable for Pi3k-Akt signaling in thymic T cells. Thymic T cells from pups at postnatal day 2 were collected. Levels of P-Akt/Akt, p-GSK3/GSK, and p-mTOR were analyzed by western blotting.

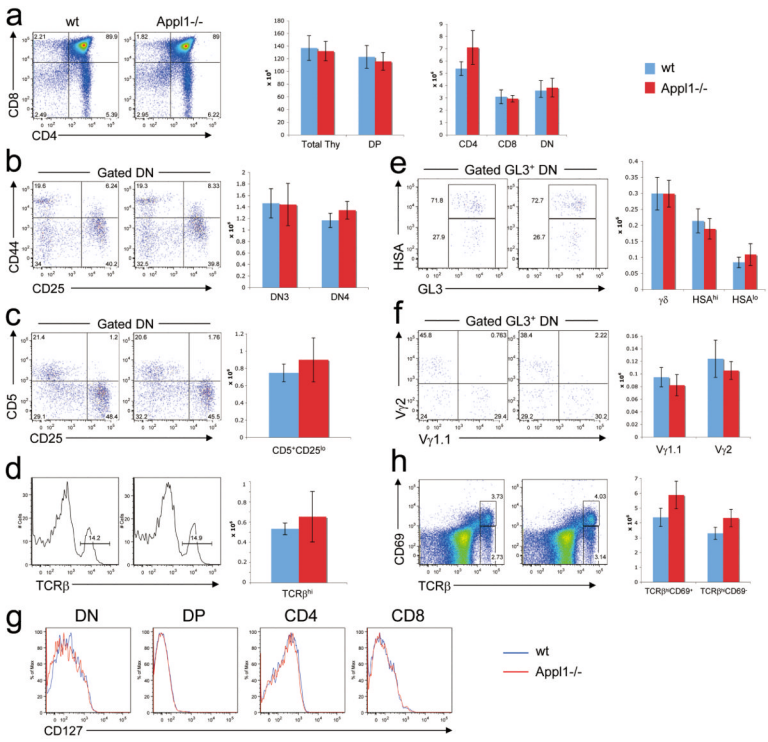

Figure 5.

Loss of *Appl1* does not affect T cell distribution in blood. Blood was drawn from 3-month-old mice. Peripheral lymphocytes were analyzed for T cell marker CD3 and B cell marker CD19 (a), TCR α/β and γ/δ (b), and CD4 and CD8 (c).

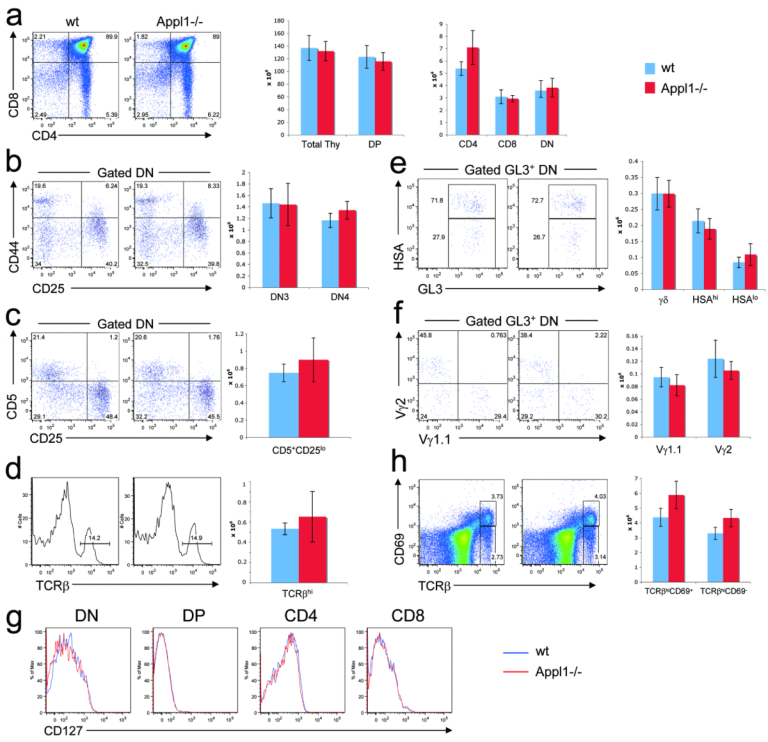

Figure 6.

Appl1-deficiency has no obvious effect on T cell development. Single cell suspensions of thymic explants from *Appl1*-deficient mice as well as age- and sex-matched littermate controls were stained with the indicated fluorochrome-conjugated antibodies and analyzed by flow cytometry. Gate frequencies are listed on the histograms. The mean ± S.D. of the absolute numbers of the indicated thymocyte subsets are represented graphically to the right.

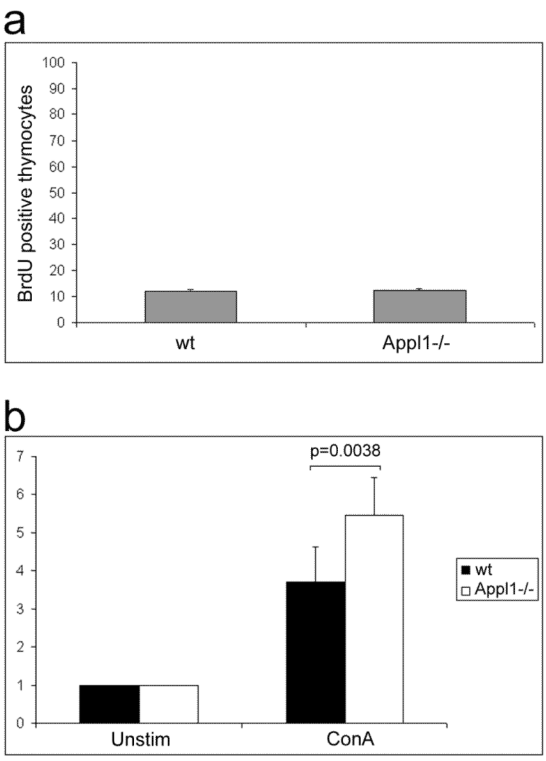

Figure 7.

Effect of *Appl1*-deficiency on T cell proliferation. (a) Primary thymocytes were incubated with 10 μM BrdU under normal culture conditions for 12 h. Cells were then stained with FITC-conjugated anti-BrdU antibody and analyzed by using FACS. (b) Primary splenic T cells were isolated using T cell enrichment columns and stimulated with ConA for 4 days. MTS assay was used to analyze cell viability/proliferation.

Table 1

Genotypic analysis of offspring from 25 Appl1+/− × Appl1+/− crosses

Gender	Wild-type	Heterozygous (+/−)	Homozygous (−/−)
Female	25 (26.3%)	47 (49.4%)	23 (24.2%)
Male	24 (23.5%)	50 (49%)	28 (27.5%)
Combined	49 (24.8%)	97 (49.2%)	51 (25.9%)