Expression and Functions of Dab2 in the Mouse Model

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UNIVERSITY OF MIAMI

EXPRESSION AND FUNCTIONS OF DAB2 IN THE MOUSE MODEL

By

Wensi Tao

A DISSERTATION

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EXPRESSION AND FUNCTIONS OF DAB2 IN THE MOUSE MODEL

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Disabled-2 (Dab2) is a widely expressed endocytic adaptor with a phosphotyrosine-binding domain (PTB) that binds to NPXY motifs found in many transmembrane receptors. Dab2 also contains a proline-rich domain that binds to Grb2 in competition with Sos1, thus Dab2 modulates Ras/MAPK activation. We produced Dab2 conditional knockout mice using Sox2-cre to delete the floxed dab2 allele. Both copies of the dab2 gene were efficiently deleted in the embryos but not in the extraembryonic endoderm. The Dab2 conditional knockout mice were viable and grossly normal. However, our investigation of these mutant mice revealed multiple biological deviations. Firstly, Dab2-deficient mammary glands showed a delay during involution. Dab2 protein was induced during lactation and by estrogen, progesterone, and prolactin in primary mouse mammary gland epithelial cell cultures. Dab2 null mammary epithelial cells were insensitive to growth suppression induced by TGF-beta. Dab2 deletion did not affect Smad2 phosphorylation and activation, but rather TGF-beta-stimulated MAPK activation was enhanced. Secondly, the Dab2-deficient mice were found lean and resistant to a high caloric diet-induced obesity. The impact of Dab2 was only found in young mice; older wild-type and Dab2-deficient mice showed no significant difference in weight gain after being
switch to a high caloric diet. In Dab2-deficient mice, the size of adipocytes was enlarged to the same extent as the wild-types, but the number of adipocytes was reduced. Dab2-deficient embryonic fibroblasts and mesenchymal stromal cells showed a reduced ability to differentiate into adipocytes. The mechanism appears related to the regulation of MAPK/Erk1/2 activity by induced Dab2 protein during adipogenic differentiation, which subsequently impacts the phosphorylation and nuclear localization of PPAR-gamma. Thirdly, Dab2 and ARH have homologous functions in LDLR endocytosis. ARH is primarily expressed in the liver and Dab2 is widely distributed throughout the body. We generated compound knockout mice for both Dab2 and ARH. The double knockout mice were viable and grossly normal. When these mice were challenged with high sucrose diet, we found that double knockout mice had a synergistic increase in serum cholesterol and LDL levels comparable to LDL receptor knockout mice. The double knockout mice also developed hepatic steatosis when fed with a high sucrose diet. In the mouse embryonic fibroblasts, we found that in the ARH-/- background, Dab2 depletion greatly reduced LDL uptake, to the level comparable to those of LDLR-/- . We conclude that Dab2 plays a compensatory but not redundant role in LDLR endocytosis in the peripheral tissues while ARH is mainly responsible for those in the liver. In summary, mammary glands in Dab2-deficient mice were delayed during involution stage. Dab2 deficient mice were resistant to high caloric diet-induced obesity. Compound knockout mice for both Dab2 and ARH have a synergistic increase in cholesterol and LDL levels. These data suggest that Dab2 functions as a cargo-selective endocytic adaptor as well as a signaling molecule negatively regulating MAPK pathway.
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<th>Description</th>
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<tbody>
<tr>
<td>AP-2</td>
<td>Adaptor Protein-2</td>
</tr>
<tr>
<td>ARH</td>
<td>Autosomal Recessive Hypercholesterolemia</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT-enhancer-binding proteins α</td>
</tr>
<tr>
<td>Cre</td>
<td>Causes Recombination</td>
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<tr>
<td>CME</td>
<td>Clathrin-Mediated Endocytosis</td>
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<tr>
<td>CLASP</td>
<td>Clathrin-Associated Sorting Proteins</td>
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<td>Colony-stimulating Factor 1</td>
</tr>
<tr>
<td>Dab1</td>
<td>Disabled-1</td>
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<tr>
<td>Dab2</td>
<td>Disabled-2</td>
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<tr>
<td>DBP</td>
<td>Vitamin-D binding protein</td>
</tr>
<tr>
<td>EH</td>
<td>Eps15 homology</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FH</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>Flox</td>
<td>LoxP-flanked</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange Factor</td>
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<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wild association studies</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>Lox P</td>
<td>Locus of X-over P1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MOSE</td>
<td>Mouse Ovarian Surface Epithelial</td>
</tr>
<tr>
<td>NPXY</td>
<td>Asn-Pro-X-Tyr</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
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<td>PTB</td>
<td>Phospho Tyrosine Binding</td>
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<tr>
<td>PtdIns(4,5)P2</td>
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<td>RAS</td>
<td>Rat Sarcoma</td>
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<td>Retinol binding protein</td>
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<td>Son of sevenless homolog 1</td>
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<tr>
<td>SRC</td>
<td>Schmidt-Ruppin A-2 Viral Oncogene Homolog</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>Transforming growth factor-beta</td>
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Chapter I: INTRODUCTION

1.1 Background of Disabled-2

The Disabled gene was first discovered in Drosophila. In the flies, Disabled can suppress the mutant phenotype of a tyrosine kinase abl in a dosage dependent manner, thus it is named Dis-abl-ed. (Gertler, Bennett et al. 1989) Since the expression and functions of Disabled and abl are closely related, Disabled was thought to be a key component of the abl signaling pathway to regulate cell migration and morphogenesis. (Gertler, Hill et al. 1993) However, 10 years later, the mutation described as Disabled was proved to be mutations in the loci of the nearby gene Nrt, which suggested the functions reported as Disabled are actually attributed to Nrt (Liebl, Rowe et al. 2003) Meanwhile, the newly generated Disabled mutations are able to produce very similar phenotypes to those mutations of the abl signaling pathway, which suggests Disabled is still a bona fide component of the abl signaling pathway. (Song, Kannan et al. 2010) Although the relationship between disabled and abl are controversial, the nomenclature of the Disabled gene is still been well accepted.

Disabled proteins are evolutionary conserved across the species. By using multiple sequence alignment, the sequences of 12 of Disabled proteins from different model organisms are compared to draw a phylogenetic tree. (Figure.1) Disabled protein homologues can be found in most multicellular organisms from nematodes to vertebrates. Interestingly, the functions of Disabled proteins are also evolutionary conserved. In C. elegans, the function of the only Disabled homologue, Dab1 is involved in lipoprotein receptor-mediated endocytosis and uptake of yolk protein. (Holmes, Flett et al. 2007) In mammals, there are two Drosophila Disabled homologous proteins: Dab1 and Dab2.
Fig. 1. Neighbor-joining phylogenetic tree of Disabled proteins.

The sequences of 12 Disabled family proteins from model organism are compared by using multiple sequence alignment tool ClustalX. The N-J phylogenetic tree was drawn by TreeView. The scale bar indicates the average amino changes per site.
Disabled-1 is mainly restricted in the brain and Disabled-2 is wildly expressed. (Howell, Hawkes et al. 1997, Xu, Yi et al. 1998) The murine Disabled-2 was first cloned as a 96 KD protein rapidly phosphorylated in macrophages cell lines in response to the mitogen stimulation of Colony-stimulating Factor 1 (CSF-1). (Xu, Yang et al. 1995) Meanwhile, the human Disable-2 was also found as a differentially expressed gene in human epithelial ovarian cancer. (Mok, Wong et al. 1994)

1.2 Domain structure of Disabled-2

The biological function of Dab2 can be revealed from the domain structure and amino acid sequences. There are two main functional domains: phosphotyrosine-binding domain (PTB) in the N-terminal and proline-rich domain (PRD) in the C-terminal. In additional to that, there are several protein-protein interaction motifs, including clathrin binding box, DPF, NPF motifs as well as myosin VI binding motifs. (Figure 2. A)

The PTB binding domains are highly conserved in evolution. PTB domain containing protein can interact with cell surface receptors with C-terminal Asn-Pro-X-Tyr (NPXY) motif via its PTB domain. The PTB domain of Dab2 preferentially interact NPXY motif in the non-phosphorylated state. The PTB domain of Dab2 is also able to interact with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) via distinct site from the NPXY motif binding site. (Yun, Keshvara et al. 2003)

Unlike PTB domain, the proline rich domain of Dab2 is diverse across the species with many protein-protein interaction motifs dispersing within this domain. By sequence alignment, the C-terminal proline rich domain of Dab2 is similar to those found SOS,
which is a Ras guanine nucleotide exchange factor (GEF). The proline-rich domain of SOS is able to interact with SH3 domain of Grb2, an adaptor protein function link SOS to receptor tyrosine kinase. By competing with SOS for Grb2 binding, the proline rich domain of Dab2 can interrupt the SOS-Grb2 complex, which may eventually lead to attenuation of Ras/MAP kinase pathway (Figure2.B). (Xu, Yi et al. 1998)

In additional to the two major functional domains, Dab2 also contains multiple protein-protein interaction motifs. P96 isoform of Dab2 contains clathrin binding boxes, NPF motifs which interact with Eps15 homology (EH) domains containing proteins Eps15, DPF motifs which can binds to AP2. These features suggest Dab2 to be an adaptor protein for clathrin-mediated endocytosis (CME). In the very C-terminus there is a myosin VI binding motifs. Structural biology data indicated that the myosin VI binding motif of Dab2 alone can induce the dimerization of myosin VI. Dab2 is cargo-specific endocytic adaptor functionally links cell surface receptors and clathrin coated vesicles with actin based endocytic machineries.

Domain organizations of Dab2 can reveal its biological activities. As an endocytic adaptor, Dab2 participates in different biological processes, such as clathrin mediated endocytosis, cell migration, nutrient update as well as epithelial organism.
There are two major alternative spicing forms of mouse Dab2 protein: the P97 and P67. The functional domains and interaction motifs of mouse Dab2 protein are illustrated (A). The proline rich domain of Dab2 can interrupt the SOS-Grb2 complex, which negatively regulate Ras/MAP kinase pathway (B).

Figure. 2. Domain structures of mouse Disabled-2 proteins. There are two major alternative spicing forms of mouse Dab2 protein: the P97 and P67. The functional domains and interaction motifs of mouse Dab2 protein are illustrated (A). The proline rich domain of Dab2 can interrupt the SOS-Grb2 complex, which negatively regulate Ras/MAP kinase pathway (B)
1.2.1 Dab2 as an endocytic adaptor

Endocytosis is a cellular process that is important for nutrient uptake, cellular homeostasis maintenance and signaling pathway controls. The major and best characterized route for endocytosis is facilitated by the clathrin-coated vesicles. After a ligand binds to the cell surface receptor, clathrin is recruited to the coated pits which subsequently bud into the cytoplasm to form clathrin-coated vesicles. (Goldstein, Brown et al. 1985) By doing so, cells are able to uptake a small area of cell surface and a small portion of surrounding liquid. (Traub 2009)

The specificity of the clathrin-mediated endocytosis is determined by cell surface receptors. For example, cell surface receptor low-density lipoprotein (LDL) receptor specifically interacts with LDL particle and mediates its endocytosis. (Goldstein, Brown et al. 1985) The signaling motif on the c-terminals of cell surface receptor consists of a short, consensus array of amino acid residues. For LDL receptor, Asn-Pro-X-Tyr (NPXY) motif is required for LDL particle internalization. Specific endocytic adapter are required for recognition of the signaling motifs. Dab2, one of the clathrin-associated sorting proteins (CLASPs), is able to interact with NPXY motif via its PTB domain. (Maurer and Cooper 2006)

There are three functional groups of PTB domains according to their NPXY motif binding capacity in different phosphorylation states. Dab2 can only interact with LDL receptors in the non-phosphorylated form. (Yun, Keshvara et al. 2003) Similarly, PTB domain of Dab2 can also interact with other LDL receptor family cell surface receptors, such as LDL receptor Related proteins (LRPs), integrin β, and β-amyloid precursor
protein. (Figure 3. A) The diversity of Dab2 interacting cell surface receptors enable Dab2 to impact on various cellular signaling pathways.

As clathrin-associated sorting proteins (CLASPs), Dab2 and Autosomal recessive hypercholesterolemia (ARH) can both facilitate the endocytosis low-density lipoprotein receptor (LDLR). Dab2 can facilitate the clathrin mediated endocytosis independent of Adaptor protein-2 (AP-2). (Figure 3. B) In the absent of Dab2, ARH requires AP-2 to mediate the endocytosis of LDLR. (Maurer and Cooper 2006) The familial hypercholesterolemia (FH) is due to mutations in gene LDLR with phenotypes of elevated serum cholesterol level. ARH mutations have similar but less severe phenotypes compared with the FH mutations (Jones, Hammer et al. 2003). These data suggested that Dab2 and ARH have homologous roles in receptor-mediated low-density lipoprotein uptake.

Integrins are cell surface receptors for various extracellular matrix components. Integrins are heterodimers consisted by different α and β subunits. The most commonly expressed β subunits-integrin β1 contains a NPXY motif which is able to interact with PTB domain of Dab2. (Prunier and Howe 2005) From the functional proteomic screening, Dab2 depletion can inhibit the cell migration by disrupting internalization β-1 integrins. (Teckchandani, Toida et al. 2009) Independent study by depletion both clathrin adaptors can block the internalization of internalization β-1 integrins as well as impair the focal adhesion disassembly, which eventually lead to the inhibition of cell migration. (Chao and Kunz 2009)

Cell lines with Dab2 mutation are founded to be deficient for TGF-beta (Transforming
growth factor-beta) signaling by genetic screening. Transfection of wild type Dab2 cDNA can restore the normal response to TGF-beta. (Hocevar, Smine et al. 2001) Dab2 can interact with TGF-beta receptor and functionally link the receptors to the downstream signaling molecules SMAD 2 and 3. Dab2 depletion can abrogate the TGF-β receptor recycling and diminish the SMAD phosphorylation. (Penheiter, Singh et al. 2010) More recent literature suggests Dab2 is a molecular switch for TGF-beta response. Dab2 deletion can abrogate the tumor suppressor function and enhancing the tumor promoter activity of TGF-beta. (Hannigan, Smith et al. 2010) Dab2 deletion does not affecting the SMAD phosphorylation, leaving the canonical TGF-beta signaling pathways intact. (Hannigan, Smith et al. 2010, Martin, Herbert et al. 2010) However, loss of Dab2 can lead to the activation of one of the non-canonical TGF-beta pathways, the Ras/MAPK (Rat Sarcoma/Mitogen-activated protein kinase) signaling pathway. (Tucker, Mackie et al. 2004)

As an endocytic adaptor, Dab2 can influence on many cellular processes such as cell migration via β1 integrin, nutrient uptake through megalin (LRP2), cell growth by TGF-beta receptor and LDL uptake with LDL receptor. By regulation of different cell surface receptors, Dab2 can have diverse biological functions in many different systems.
Figure 3. Structure and functions Disabled-2 as an endocytic adaptor. X-ray crystallographic structure of Disabled-2 PTB domain interacts simultaneously with NPXY motif of the β-amyloid precursor protein (APP) peptide (Green) and PtdIns(4,5)P2 (Red) attached to cell membrane. (Yun, Keshvara et al. 2003) (A) Schematic diagram of Dab2 facilitating the LDL uptake by clathrin mediated endocytosis. (B)
1.3 Dab2 in mouse development

1.3.1 Dab2 in mouse embryonic development

Mouse embryos are developed from fertilized eggs. After fertilization, the zygotes undergo several rounds of cell division and develop into blastocysts. At around E4.5 stage, cells within the blastocyst segregated into two lineages: the epiblast which will give rise to the embryo proper and primitive endoderm which will become extra embryonic tissues. (Rossant and Tam 2009) Upon hatching, the primitive endoderm developed into visceral endoderm and parietal endoderm surrounding the epiblast. Visceral endoderm is a layer of coherent and endocytically active cells required for nutrient uptake and waste exchange for the epiblast. (Bielinska, Narita et al. 1999) The lineage of the primitive endoderm is driven by GATA family transcriptional factors. The primitive endoderm specific genes, such as Dab2 and laminin, induced by the GATA factors, can facilitate cell sorting by modulate cell surface adhesion and polarity. (Chazaud, Yamanaka et al. 2006, Yang, Cai et al. 2007)

Disabled-2 plays an important role in murine embryonic development. Two independent gene knock out studies have demonstrated that Dab2 constitutive knockout mice are embryonic lethal. (Morris, Tallquist et al. 2002, Yang, Smith et al. 2002) During early embryonic development, expression of Dab2 is detected in extra-embryonic endoderm but not in epiblast. In Dab2 null mice, extra-embryonic endoderm is disorganized and intermixed with ectodermal cells in E5.5 stage.(Moore, Cai et al. 2013) In the endodermal cell, Dab2 can mediate the directional endocytosis, which leads to the establishment of an apical polarity to form a coherent primitive endoderm layer. In the absent of Dab2, the
apical polarity is lost, and finally leads to the failure of surface positioning of primitive endoderm. (Yang, Cai et al. 2007) Moreover, deletion of c-fos, one the downstream signaling molecules of MAPK/ERK (Mitogen-activated protein kinase/Extracellular signal-regulated kinases) pathway, can partially rescue the knockout phenotypes of Dab2 deletion. These data suggested Dab2 impacts the primitive endoderm organization partially by influencing the MAPK/ERK pathway. (Yang, Smith et al. 2009)

1.3.2 Dab2 in adult development

Although Dab2 is required for the murine embryonic development since it essential role in primitive endoderm function, it seems dispensable for adult development. To achieve the deletion of Dab2 gene only in the embryo proper but leave the extra embryonic tissue intact, a conditional knockout mice were made with crossing Dab2 f/f/ female mice with Sox-2Cre; Dab2 DF/+ male mice. The resulting mice inherited with genotype Sox-2 Cre/ Dab2 DF/F can effectively delete Dab2 gene in the whole body. Dab2 deficient mice are grossly normal with no obvious defects other than mild proteinuria. (Morris, Tallquist et al. 2002, Moore, Cai et al. 2013)

The lack of major adult phenotypes of Dab2 deficient mice may due to compensatory expression of other PTB domain containing proteins, such as Numb and ARH (Autosomal Recessive Hypercholesterolemia). During early embryonic development, ARH expression is found in parietal endoderm but absent in visceral endoderm. (Maurer and Cooper 2006) Lack of ARH in visceral endoderm suggests that Dab2 may be the only PTB domain containing adaptor in the visceral endoderm. However, in the adult tissue, expression of Numb and ARH may functionally compensate for the endocytic activity of Dab2.
1.4 Expression of Dab2 in different tissues

Dab2 expression is wildly found in several tissues types. Dab2 is widely expressed in surface epithelium, such as kidney (Morris, Tallquist et al. 2002), macrophages (Rosenbauer, Kallies et al. 2002), lactating mammary gland (Schwahn and Medina 1998), placenta and ovary (Fazili, Sun et al. 1999). From the online High-throughput gene expression profiling database, we have found that Dab2’s expression in various tissues (Figure 4).

1.4.1 Expression and function of Dab2 in the kidneys

Dab2 expression is found in proximal tubule of murine kidney (Morris, Tallquist et al. 2002). The main physiological function of proximal tubule is reabsorption of small molecular weight protein such as albumin, which is facilitated by receptor mediated endocytosis. Megalin is multiligand endocytic receptor for small molecular weight proteins such as albumin, vitamin-D binding protein (DBP), retinol binding protein (RBP). The cytoplasmic tail of megalin contains a NPXY motif which binds to Dab2 (Gallagher, Oleinikov et al. 2004). The expression of both Dab2 and megalin are found in the proximal tubular brush border. In the Dab2 conditional knockout mice, due to the suboptimal transportation of megalin, the subcellular localization of megalin is redistributed from endosomes to microvilli, along with the reduced megalin protein level (Nagai, Christensen et al. 2005). Furthermore, reduced transportation of megalin in Dab2 conditional knockout mice also lead to increase urine level of RBP and DPB (Morris, Tallquist et al. 2002). Recently, genome wild association studies (GWAS) have found that Dab2 loci is associated with chronic kidney disease (Boger and Heid 2011, Chasman, ...
Fuchsberger et al. 2012) In the proximal tubule, Dab2 plays an important role in the endocytosis of cell surface receptor megalin, which is a multiligand receptor for reabsorption of small molecule proteins such as RBP and DPB.

1.4.2 Expression and function of Dab2 in macrophages

Dab2 is originally cloned as a phosphoprotein regulated by Colony-stimulating Factor 1 in a murine macrophages cell line. (Xu, Yang et al. 1995) Dab2 expression has been found in the macrophages in the adult tissue. (Cheung, Mok et al. 2008) In the macrophages, cell adhesions can induction Dab2 phosphorylation and its association with cytoskeleton and cell membrane. In the same time, induction of Dab2 protein can accelerate cell adhesion and spreading. (Rosenbauer, Kallies et al. 2002) In the disease model of multiple sclerosis, Dab2 is induced in lesional macrophages in the murine spinal cord. (Jokubaitis, Gresle et al. 2013) Thus, Dab2 functions as an inducer of cell adhesion which can facilitate the cell spreading and migration in the macrophages.
Figure 4 High-throughput gene expressions profiling of Dab2 in different tissues. Microarray dataset is acquired from online dataset GeneAtlas MOE430, gcrma (http://biogps.org/#goto=genereport&id=13132). The final readouts are averaged by four different probe sets and compiled according to Dab2 mRNA expression levels.
1.5 Dab2 as a putative tumor suppressor

1.5.1 Loss of Dab2 expression in cancers

Using DNA-fingerprinting approach to detect differential gene expression in ovarian cancer cells lines, cDNA fragments of Dab2 have been found differentially expressed in ovarian cancer. Dab2 mRNA were present in normal ovarian surface epithelial cells but consistent absent in the ovarian cancer cell lines. (Mok, Wong et al. 1994) Additional immuno-histological chemistry and cell culture experiments further suggested Dab2 to be a tumor suppressor in ovarian cancer. (Mok, Chan et al. 1998, Fazili, Sun et al. 1999) Moreover, Dab2 was also found down regulated in rodent mammary tumors, suggesting Dab2 also plays a role in breast cancer. (Schwahn and Medina 1998) Subsequently, numerous studies have confirmed that loss of Dab2 expression in human mammary cancer.(Sheng, Sun et al. 2000, Bagadi, Prasad et al. 2007) IHC staining has indicated that Dab2 expression is lost in the majority of the human breast and ovarian tumors, and it is an early event in tumorigenicity. Loss or reduced Dab2 expression were also been detect in many other types of epithelial cancer, including prostate, colon, lung and head and neck cancers.(Hannigan, Smith et al. 2010)

1.5.2 Dab2’s functions as a tumor suppressor

Dab2 functions in endocytic trafficking to maintain cell polarity and epithelial organization. (Yang, Cai et al. 2007) Loss or reduction of Dab2 expression leads to the anchorage independent proliferation of breast and ovarian cancer cells. Transfection of Dab2 into ovarian and breast cancer cells lacking Dab2 expression can restore the requirement of adhesion to basement membranes.(Sheng, Sun et al. 2000) Thus, thus loss
of Dab2 may lead to epithelial disorganization anchorage-independent proliferation in mammary and ovarian epithelial cells.

In addition to maintaining epithelial organization, Dab2 also modulate several important signaling pathways. Dab2 is required for transmission of TGF-beta signaling by functionally link TGF-beta receptors and the SMAD family proteins. (Hocevar, Smine et al. 2001) Additional studies suggested detailed mechanisms for the function of Dab2 in regulating TGF-beta pathways. (Hocevar, Prunier et al. 2005, Prunier and Howe 2005, Martin, Herbert et al. 2010) In head and neck cancer, loss of Dab2 compromised the tumor suppressor function of TGF-beta, while enabling its tumor promoting activities. (Hannigan, Smith et al. 2010) Thus, Dab2 is proposed to be a molecular switch for TGF-beta from a potent tumor suppressor to a tumor promoter.

The prolin-rich domain in the C-terminus of Dab2 shares a great homology to the proline-rich domain in Sos, a guanine nucleotide exchange factor for Ras. (Xu, Yi et al. 1998) Two SH3 domains of Grb2 can interact with Sos vis the proline-rich domain, linking Sos with Ras. Both Sos and Dab2 bind competitively to the SH3 domain Grb2. (Xu, Yi et al. 1998) Therefore, by compete with Sos for binding to Grb2, Dab2 cam attenuate the association between Sos and Grb2 as a negative regulator for Ras/MAPK pathway.

1.6 Summary and significance

In this thesis, I aim to analyze the expression and functions of Dab2 in the mouse model. Previous researches of Dab2’s are mainly performed in in vitro cell cultures system. Some of the reports on Dab2’s functions are controversial or even contradictory. The physiological functions of Dab2 in vivo remain largely unknown. My study using Dab2
conditional knockout mice not only provide an *in vivo* verification of current *in vitro* studies, but also give an insight into physiological functions of Dab2 *in vivo*.

As an endocytic adaptor and a signaling molecule, I have demonstrated that Dab2 null mice have several adult abnormalities. Firstly, Dab2-deficient mammary glands showed a strikingly delayed during involution stages because of delayed cell death and apoptosis. Secondly, Dab2-deficient mice are lean and resistant to high caloric diet-induced obesity. Dab2 deficient MEF cells are impaired in adipogenic differentiation. Thirdly, I have generated compound knockout mice for both Dab2 and ARH. The double knockout mice are viable and grossly normal. When these mice are challenged with high sucrose diet, we found that double knockout mice have a synergistic increase in cholesterol and LDL levels comparable to LDL receptor knockout alone. Together, this information will advance our understanding of physiological functions of Dab2 the mouse models.
Chapter II: THE ROLE OF DAB2 IN MAMMARY GLAND DEVELOPMENT

As a putative tumor suppressor, Dab2 expression is lost in breast cancer. (Schwahn and Medina 1998) In breast cancer cell lines, loss or reduction of Dab2 expression leaded to the anchorage independent proliferation. Transfection of Dab2 into breast cancer cells loss Dab2 expression can restore the requirement of attachment to basement membranes. (Sheng, Sun et al. 2000) In mouse mammary gland epithelial cells lines, si-RNA mediated suppression of Dab2 prevents TGFβ-induced EMT and promotes TGF-β-induced apoptosis. (Prunier and Howe 2005) Although the roles of Dab2 in cancer development are well studied, the physiological functions of dab2 in mammary gland are still largely unknown. In this chapter, I will focus on the expression and function of Dab2 during different developmental stages in murine mammary gland development.

In this study, we found Disabled-2 expression mainly present in murine mammary gland epithelial cells. Expression of Dab2 was correlated with different developmental stages during pregnancy cycles. In breast epithelial cells, dab2 expression was induced during pregnant stage and further increased during lactating stages. Since mammary development is tightly regulated by developmental hormones, Dab2 expression may be controlled by hormones during pregnant cycles.

To investigate the physiological roles of Dab2 in mammary gland development, a mutant line of mice with Dab2 gene deletion was generated to bypass early embryonic lethality. Dab2-deficient mammary glands were delayed during involution stage. Dab2 protein levels can be induced by estrogen, progesterone, and prolactin in primary mouse mammary gland epithelial cell cultures. Dab2 null mammary epithelial cells are
insensitive to growth suppression induced by TGF-beta. Nevertheless, Smad2 phosphorylation was not affected by Dab2 deletion. TGF-beta stimulated MAPK activation was elevated in Dab2 null cells. In summary, Dab2 expression is controlled by reproductive hormones and Dab2 is involved in apoptotic clearance of breast epithelial cells during involution stage by modulating TGF-beta pathway.

2.1 Induction of Dab2 expression in mammary glands

Previous unpublished data from our lab have found Dab2 expression in mammary glands varied greatly in different physiological stages. In virgin mice, the Dab2 protein was undetectable in mammary epithelial cells by immunohistochemistry (Figure 5A, arrow), while Dab2 staining was uniform and intense in all mammary epithelial cells (arrow) of the mammary glands during lactation stage (Figure 5B, arrowhead). Immunofluorescence staining of Dab2 indicated Dab2 mainly localized in the apical surface of epithelial cells (Figure 5C). The induction of Dab2 protein in mammary glands was verified by Western blot analysis of tissue lysates (Figure 5D). While Dab2 was undetectable in virgin mammary glands, a low level appeared during pregnancy (15.5 days), and several isoforms, including p96 and p67, were massively induced upon lactation. In the involuting mammary glands, Dab2 proteins were lost (Figure 5D). Mammary tissue lysates from Dab2 conditional knockout mice were used to distinguish Dab2 isoforms from non-specific signals in the Western blots. Since mammary tissues contain multiple cell types, such as stromal, adipocytes, and immune cells, in addition to epithelial cells, we assayed E-cadherin as an indicator of epithelial content (Figure 5D). Beta-catenin was
also probed, and it inversely correlated with E-cadherin levels. Based on equal protein
loading and an equivalent beta-actin signal, the fraction of mammary epithelial cells was
low in virgin, increased and remained similar in pregnant and day 1 lactating, and was
highest in day 5 lactating mice (Figure 5D). These results indicated that in mammary
glands, Dab2 protein expression was induced in epithelia following pregnancy, reached
maximum during lactation, and waned upon involution.

From these data, we can conclude that Dab2 isoforms mainly express in the epithelial
structures in the mammary gland. Dab2 expression is correlated with lactogenic
differentiation of mammary gland epithelial cells. In the virgin mammary gland, in the
undifferentiated terminal end buds, we hardly detect any Dab2 protein by
immunohistochemistry. However, Dab2 expression emerges during mid gestation when
mammary epithelial cells start to differentiate into mature mammary gland producing
milk components. Furthermore, the amount of Dab2 protein isoforms reached maximum
during lactation, when milk components are secreted by a process called exocytosis.
However, Dab2 protein levels also decreased during involution, when the mammary
glands undergo massive apoptosis and remodel. These data suggested Dab2 may involve
in milk secretion and exocytosis during lactation.
Figure 5. Induction of Dab2 by pregnancy and lactation in mammary glands. Mammary glands from virgin (A) and lactating (B) wildtype mice were harvested and used for histological (A,B) and immunofluorescence(C) analysis. Representative images of Dab2 immunostaining are shown. Arrows indicate the mammary epithelial cells. (D) The induction of Dab2 proteins was confirmed by Western blot. Mammary tissues from conditional Dab2 knockout mice (dab2 (f/f):Meox2-Cre) (cKO) were used as controls for antibody specificity. The tissues were lysed for Western blot analysis of Dab2, beta-catenin, and E-cadherin. Arrows indicate Dab2 p96 and p67 isoforms and a band that is presumably IgG heavy chain.
2.2 Induction of Dab2 expression in mammary epithelial cells by reproductive hormones

Because mammary gland development during pregnancy cycle is tightly orchestrated by female reproductive hormones, we speculated that differential Dab2 expressions in mammary epithelial cells might be regulated by reproductive hormones. We tested this hypothesis with primary mouse breast epithelial cells in cultures (Figure 6). In mouse breast epithelial cells isolated from virgin mammary glands, only progesterone can induced an about 4 folds increase in Dab2 proteins (Figure 6A, B). When applied together, progesterone and prolactin can achieve a synergistic induction of Dab2 protein to about 10 folds (Figure 6A, B). In order to isolate sufficient number of cells for additional experiment, the primary mouse mammary epithelial cells were isolated from mice in the mid stage of pregnancy, and these preparations contained more than 90% cytokeratin-positive epithelial cells. In these primary cell cultures Dab2 protein were regulated by physiological levels of estrogen, progesterone, and prolactin, while the combination of progesterone and prolactin together was the most potent inducer of Dab2 expression (Figure 6C). Several Dab2 isoforms, including the p96 and p67, were both induced after exposure to hormones for 4 days. Mammary epithelial cells harvested from Dab2 conditional knockout mice were used as negative controls for the specificity of Dab2 proteins in Western blot. The maximal induction of Dab2 proteins by both prolactin and progesterone was estimated to be 22-fold (Figure 6D).
Figure 6. Hormonal induction of Dab2 expression in primary mammary epithelial cells. Mammary epithelial cells were prepared from wildtype control (fl/+) and Dab2 conditional knockout (df/df) mice inheriting Meox2-Cre. The cells were treated with 17 beta estradiol (1 nM), progesterone (1 µM), and prolactin (1 nM, or 50 ng/ml), individually or in combination for 4 days in culture. (A) Dab2 and beta-actin from primary mammary epithelial cells of virgin mice were analyzed by Western blot. (B) The signals of Dab2 proteins from the Western blot were quantified using NIH Image J software using beta-actin as normalization control. Relative values were plotted with the value of untreated cells defined as 1.0. (C) Dab2 and beta-actin from primary mammary epithelial cells isolated from pregnant mice were analyzed by Western blot. Dab2 protein was absent in cells from the conditional knockout mice. (D) The relative intensity of Dab2 proteins on Western blots was quantified using NIH Image J software, using beta-actin for normalization.
2.3 Mammary glands in mosaic Dab2 conditional knockout mice

To bypass early embryonic lethality (Morris, Tallquist et al. 2002, Yang, Smith et al. 2002), a mutant line of mice with Dab2 gene deletion was generated to study the roles of Dab2 in adult mammary gland. (Moore, Cai et al. 2013) In this novel mouse model, both Meox2-Cre (Tallquist and Soriano 2000) and Sox2-Cre (Hayashi, Tenzen et al. 2003) can effectively delete Dab2 gene in embryonic proper but leave extraembryonic tissues intact. The resulting Dab2 conditional knockout mice were grossly normal without major developmental defects. (Moore, Cai et al. 2013) In the adult lactating mammary gland, the resulting dab2 knockout mammary glands were grossly normal compared with control mammary glands. By Immunohistochemistry staining using Dab2 antibody, the estimated efficiency for Dab2 gene deletion was about 95% in dab2 (f/df): Meox2-Cre with remaining clustered Dab2 positive epithelial cells (Figure 7A). Moreover, there was essentially no Dab2 positive cells found in dab2 (f/df): Sox2-Cre mammary glands because of the highly efficient deletion by Sox2-cre in adult tissues. (Figure 7B). The degree of deletion efficiency by both Meox2-Cre and Sox2-Cre correlated with PCR genotyping result of genomic DNA extracted from tail. (Figure 7C)

Virgin female mammary gland from Dab2 knockout, heterozygous and wildtype were compared, no significant morphological differences were found between these genotypes. During pregnant stage, the mammary glands of Dab2 conditional knockout mice were also able to undergo normal branching morphogenesis as the control mammary glands. Dab2 acts as an endocytic adaptor which influences uptake and secretion of small molecule protein in kidney (Morris, Tallquist et al. 2002) and transportation of vitamin D binding protein in human mammary cells. (Chlon, Taffany et al. 2008) The milk secretory
functions of Dab2 deficient mammary gland were tested. Milk was collected from Dab2 deficient and control mice. Major milk protein components were separated by SDS-PAGE and identified by Commassie staining (Figure 7D). The specific protein beta-casein and Vitamin D binding protein (GC-globin) were detected by Western blot (Figure 3E). No major differences were detected in milk contents between knockout and controls. Furthermore, Dab2 deficient female mice were capable of normal nursing of the pups. In summary, loss of Dab2 in mammary gland has minimal impact on milk secretion and nurturing litters. Furthermore, other endocytic components were examined in Dab2 deficient mammary gland. In the 5 day lactating mammary gland from mosaic knockouts mice (dab2 (f/df):Meox2-Cre), the remaining Dab2 positive cell had a more intense apical localization signaling of adaptin-alpha and clathrin (Figure 4A and B, arrow) than Dab2-negative cells (Figure 4A and B, arrowhead). These data suggested Dab2 might impact on the distribution of endocytic components. Since Dab2 also played a role in polarity maintenance and epithelial organization of primitive endoderm (Yang, Cai et al. 2007), apical and basolateral markers of mammary gland epithelial cells were examined.
Figure 7. Normal lactation in mammary glands from dab2 mosaic knockout mice. 
(A) Mammary glands from 4-month old, 5-day lactating mice of dab2 (f/df):Meox2-Cre genotype show mosaic Dab2 immunostaining. (B) Dab2 was absent in more than 99% of mammary epithelial cells from mice of dab2 (f/df):Sox2-Cre genotype. (C) DNA extracted from tail tissues was used to genotype flox (f) and delta flox (df) alleles of the dab2 gene in dab2 (+/df): Sox2-Cre, dab2 (f/df):Meox2-Cre, and dab2 (f/df):Sox2-Cre. (D) Milk was harvested from dab2 heterozygous (dab2 (+/df):Sox2-Cre) and knockout (dab2 (f/df):Sox2-Cre) mice. Total proteins were resolved by SDS-PAGE and stained with Coomassie blue. (E) Specific proteins, beta-casein and GC-globin (vitamin D binding protein), were analyzed by immunoblot.
In the Dab2 deficient cells, the basolateral marker E-Cadherin were more cytoplasmic and punctate (Figure 4C, arrowhead) while a predominant basolateral in the Dab2 positive cells. Although no obvious differences were detected in the distribution of one of the apical makers, sodium/phosphate co-transporter NPT2b (Figure 4D), a loss of apical localization of another apical makers the calcium pump PMCA2 were found in the Dab2-deficient cells (Figure 4E, arrowhead) compared to the adjacent Dab2-proficient cells. No obvious changes were detected in the distribution of the apical marker, (Figure 4E, arrow). Histological sections of lactating mammary gland from mosaic knockouts mice (dab2 (f/df):Meox2-Cre) contained regions with Dab2 positive and negative cells in the same field, which made it possible to analyze phenotypical differences depending on Dab2 expression.

In summary, Dab2 loss affected the polarized distribution of certain types of endocytic components, and cell polarity markers, but did not alter the gross morphology or the lactation function of mammary glands.
Figure 8 Mild effects of Dab2 deletion on endocytic components and polarity markers in mammary epithelia. Mammary glands from lactating (5 days) mice of dab2 heterozygous controls and mosaic knockouts (dab2 (f/df):Meox2-Cre) were analyzed by confocal immunofluorescence microscopy. (A) Adaptin alpha and Dab2 double staining of mammary glands from a mosaic dab2-deleted mouse, comparing Dab2-positive (arrow) and negative (arrowhead) cells; (B) Clathrin and Dab2 co-staining of mammary glands from a mosaic dab2-deleted mouse, comparing Dab2-positive (arrow) and negative (arrowhead) cells; (C) Dab2 and E-cadherin staining: E-cadherin is localized more in the cytoplasm in Dab2-deleted cells (arrowhead); (D) NPT2b, a sodium-phosphate cotransporter; and (E) PMCA2 ATPase, a calcium pump, in Dab2-positive (arrow) and negative (arrowhead) cells.
2.4 Delayed mammary involution in Dab2 conditional knockout mice

Although Dab2’s expression was induced in pregnancy and lactation stages, the Dab2-deficient female mice were able to undergo normal gestation without any major defects. However, the Dab2-deficient mammary glands were delayed in mammary gland regression during involution (Figure 9). Forced involution was achieved by separating the pups 12 days after birth from lactating female mice. Histological sections of involution mammary glands were analyzed. At Day 2 of involution, accumulation of cells and debris were found in the alveolar lumens of Dab2 deficient mammary gland compared with control mice (Figure 9A). In the day 3 heterozygous control group, epithelial alveoli regressed greatly, while the adipose tissues repopulated the mammary glands. Meanwhile, the Dab2 deficient mammary tissues were still mostly composed of epithelial components at this stage. Images at higher magnification indicated that the lumens harbored a large number of rounded cells with condensed nuclei (Figure 5B, arrow). In control mammary gland, such cells are present but very scarce. However, at day 5, the difference between Dab2-deficient and control mammary gland are minimal, which suggested Dab2-deficient mammary glands were still able to undergo epithelial regression in mammary involution (Figure 9A, B). Furthermore, electron microscopy was used to examine the day-3 involuting mammary glands (Figure 9C). In the Dab2-deficient mammary glands, an increased number of vacuoles and condensed nuclei were observed in the interior of the lumens (Figure 59, arrow). Consistent results were obtained in two independent experiments using deletion by Meox2-Cre or Sox2-Cre. Thus, Dab2 deletion could consistently impact on mammary gland involution.
Figure 9. Dab2 deficient mammary glands have delayed involution. A control (dab2 heterozygous) and dab2 knockout (dab2 (f/df):Sox2-Cre) group of six 6-month old mice were mated, became pregnant, gave birth, and nursed an equal number (six) of pups per mouse. At day 12 of lactation, the mice underwent forced involution by removal of the pups. Mammary glands were harvested accordingly for histological analysis. Consistent results were obtained in two independent experiments using deletion by either Meox2-Cre or Sox2-Cre. (A) Representative H&E images show the morphology of the mammary glands. (B) Images of higher magnification are shown for day 3 and 5 of involution. Accumulating apoptotic cells in the lumen are indicated by a green arrow. (C) Mammary tissues of heterozygous and dab2 knockout at day 3 of forced involution were analyzed by transmission electron microscopy. An arrow indicates the presence of cells in the interior of the dab2 knockout mammary lumens.
Since the most striking difference occurred at day 3 of involution, we further characterized the phenotypes using several markers. First, we looked into the presence of macrophages which has a high level of Dab2 expression (Xu, Yi et al. 1998). These cells were able to engulf and clear dead cells and debris during involution. However, no significant differences in the F4/80 positive cell number or distribution were observed. (Data not shown)

Apoptotic and cell survival makers were further analyzed. In control day-3 involution mammary gland, cleaved caspase-3 staining was intensive which indicated activated apoptosis (Figure 10A, arrow). However, in the Dab2 deficient mammary gland, the staining appeared lighter and more diffuse, with less caspase-3-positive cells present (Figure 10A, arrowhead). The Dab2 null mammary glands have more p-Erk (Figure 10B), and Bcl-2 (Figure 10C) positive cells than control mammary glands. In Western blot analysis of involuting mammary glands, we found that at 3 day, Dab2 protein levels increased and presented as a higher molecular weight smear (Figure 10D). This smear might be modified proteins (such as ubiquitin conjugated). Dab2 p96 and p67 protein isoforms were absent by day 7 of involution. Western blots also showed the reduction of E-cadherin in both Dab2-positive and negative mammary glands, and the pro-apoptotic protein levels of Bax and activated caspase-3 were greater in controls than the Dab2-deficient tissues (Figure 10 E and F). Levels of the pro-survival proteins, particularly Bcl-2, were significantly elevated in Dab2 conditional knockout mammary glands compared to heterozygous controls (Figure 10F). Notably, we found that the phosphorylation and activation of Erk1/2, a pro-survival signal, were augmented on day 3 of involution in Dab2-deficient mammary glands (Figure 10F). On day 5, the differences
in Erk1/2 activation and expression of apoptotic regulators were diminished between Dab2-proficient and deficient mammary glands (Figure 10F). No significant difference in phospho-Smad2 was observed between Dab2-posoitive and deficient tissues (Figure 10F).

In summary, Dab2 deletion in mammary gland caused the unsuppressed Erk activation, increased pro-survival markers (such as Bcl-2), decreased apoptotic activation (such as Bax and cleaved caspase-3) and ultimately delay in cell death and clearance.

2.5 In vitro growth and signaling of dab2 knockout mammary epithelial cells
Since TGF-beta signaling is known to be critical in mammary involution and several reports suggest a role of Dab2 in the regulation of this pathway. We investigated TGF-beta signaling and growth control in primary mammary epithelial cells isolated from dab2 knockout and control mice. Unlike involution in vivo, TGF-beta failed to induce significant cell death in cultures of primary mammary epithelial cells. Nevertheless, upon TGF-beta exposure, the wildtype mammary epithelial cells showed a reduced cell proliferation (Figure 11A). However, Dab2-deficient cells exhibited an unsuppressed proliferation and were refractory to TGF-beta induced growth inhibition (Figure 11A). Dab2 deficiency did not eliminate canonical TGF-beta signaling, indicated by the phosphorylation and activation of Smad2 (Figure 11B), but led to a higher basal and TGF-beta-stimulated Erk1/2 activation (Figure 11B, C). Additionally, we observed a
Figure 10. Delayed apoptosis of Dab2-deficient mammary epithelial cells during involution. (A) The day 3 involuting mammary glands from control (dab2 heterozygous) and dab2 knockout (dab2 (f/df):Sox2-Cre) mice were analyzed. Apoptotic cell death in situ indicated by immunostaining for activated caspase-3, comparing wildtype (arrow) and Dab2 deficient cells (arrowhead). (B) A representative immunofluorescence microscopy staining for phospho-Erk1/2 in Dab2 heterozygous and knockout mammary glands on day 3 of involution. Phospho-Erk1/2 (red) overlaying on DAPI (blue) stainings are shown. (C) Immunofluorescence microscopy for Bcl-2 (red) and DAPI (blue) staining of day 3 involuting mammary glands. (D) Dab2 expression was determined by Western blot of tissue extracts of involuting mammary glands from dab2 heterozygous mice at 0, 1, 3, 5, and 7 days following forced involution. (E) Western blot analysis of E-cadherin, Bcl-2, Bcl-xl, and activated caspase-3. Mammary protein extracts from heterozygous lactating (day 12) and forced involuting (day 3) mice were analyzed. (F) Western blot analysis of Bcl-2, Bcl-xl, Bax, phospho-Smad2, total Smad2, phospho-Erk1/2, and total Erk1/2 in protein lysates extracted from mammary glands following 3 and 5 days of forced involution. Two independent samples (duplicate) of each genotype from different mice are shown in the blot.
slight increased amount of PCNA (an indicator of cell proliferation), and an increased Bcl-2 level in Dab2-deficient compared to Dab2-proficient cells (Figure 11B). Bax and activated caspase-3 levels were not significantly altered (Figure 11B), consistent with the lack of extensive TGF-beta induced apoptosis in the cultured cells. The TGF-beta signaling experiments were performed 5 times, and the results were entirely consistent. In summary, TGF-beta suppressed growth of wildtype mammary epithelial cells in vitro. However, the suppression was abolished in Dab2-deficient cells, accompanied by an increased Erk1/2 activation (Figure 11C). We further tested the molecular mechanism for the increased phospho-Erk1/2 in the absence of Dab2. Several previous studies have suggested that Dab2 binds Grb2, competing with Sos and thus suppressing the Ras/MAPK pathway. In primary mammary epithelial cells, co-immunoprecipitation was used to assay the competitive association between Grb2 and Sos or Dab2 (Figure 11D). In Dab2-positive control cells, TGF-beta stimulation led to a progressively increased association between Grb2 and Dab2 and a declining binding of Grb2 with Sos. In the absence of Dab2, persistent Grb2 and Sos interaction was maintained as shown by immuno-coprecipitation and Western blot (Figure 11D, E). Thus, the deletion of Dab2 led to an increased Grb2-Sos association and an unsuppressed TGF-beta-stimulated MAPK activation in mammary epithelial cells.
Figure 11. Dab2 modulates TGF-beta-stimulated growth regulation and signaling in primary mammary epithelial cells. Mammary epithelial cells were prepared from pregnant control (+/df) and dab2 conditional knockout (df/df) mice inheriting Sox2-Cre. The cells were treated with or without TGF-beta (10 ng/ml). (A) Growth of the cells was determined by WST assay over a 6-day period. Student-t test indicated that the difference in cell growth was statistically significant for at day 3 to 6 for dab2 (+/df) (p<0.005) but not dab2 (df/df) cells. (B) Protein lysates prepared from the primary cultures were analyzed by Western blots for Dab2, phospho-Smad2, E-cadherin, N-cadherin, phospho-Erk1/2, total Erk1/2, PCNA, Bcl-2, Bcl-xl, Bax, activated caspase-3, and beta-actin. (C) The relative protein level was quantified from the Western blots using NIH Image J software, and the values of the optical density (O.D.) critical markers (p-Smad2, p = Erk1/2, and Bcl-2) were compared. (D) Co-immunoprecipitation was performed to determine the association between Grb2 and Sos or Dab2. The primary cells were stimulated by TGF-beta for a time course of 0, 5, 15, 30, and 90 min. At each time point, the monolayer was washed with ice-cold PBS, lysed, and the post-nuclear supernatants were used for immunoprecipitation with antibodies against Grb2. The eluted proteins from the immunoprecipitation were separated by SDS-PAGE, and immunoprobed for Sos, Dab2, and Grb2. The immunoprecipitation experiments were performed twice and similar results were obtained. (E) Relative Dab2 and Sos protein
2.6 Summary and significance

The induction of Dab2 expression and the phenotype of mammary glands in Dab2 conditional knockout mice were described in this chapter. Dab2 deficiency delays epithelial cell death and clearance during mammary involution. We have provided data to suggest a working model whereby Dab2 expression is induced during lactation to modulate TGF-beta signaling by suppressing TGF-beta-stimulated MAPK activation. Dab2 retards MAPK activation by competing with Sos for binding to Grb2 and thus ultimately suppresses the signaling pathway (Figure 12).
Figure 12. Schematic illustration of a working model for Dab2 in modulation of TGF-beta pathway. Canonical TGF-beta signaling pathway consists of Smad phosphorylation and mediation of transcriptional regulation, leading to cell death and growth suppression, representing a tumor suppressor activity. The non-canonical route includes the activation of Ras/MAPK pathway as a result of phosphorylation and binding of TGF-beta receptor to Shc and consequently recruitment of Grb2 and Sos. TGF-beta-stimulated activation of Ras/MAPK pathway induces expression of genes involved in cell survival and growth. Dab2 modulates TGF-beta signaling by sequestering Grb2 from Sos, resulting in a reduction of Ras/MAPK activation yet allowing Smad-mediated gene transcription.
Chapter III: THE ROLE OF DAB2 IN ADIPOGENESIS

Disabled-2 (Dab2) is a widely expressed endocytic adaptor protein with a phosphotyrosine-binding domain (PTB) that binds to an NPXY motif found in many membrane proteins. Dab2 also contains a proline-rich domain that binds Grb2 in competition with Sos1, thus Dab2 modulates Ras/MAPK activation. Conditional Dab2 knockout mice were made using Sox2-cre to delete the flox dab2 allele. Both copies of the dab2 gene were efficiently deleted in the embryos but not in the extraembryonic endoderm. The mice bypass the early embryonic lethal phenotype found in the constitutive knockout and showed no obvious developmental phenotypes. However, the Dab2-deficient mice are lean and resistant to high caloric diet-induced obesity. The impact of Dab2 was only found in young mice; older wild-type and Dab2-deficient mice showed no significant difference in weight gain after feeding a high caloric diet. In Dab2-deficient mice, the size of adipocytes was enlarged to the same extent as the wild-types, but the number of adipocytes was reduced. Dab2-deficient embryonic fibroblasts and mesenchymal stromal cells showed a reduced ability to differentiate into adipocytes. The mechanism appears related to the regulation of MAPK/Erk1/2 activity by Dab2 induced during adipogenic differentiation, which subsequently impacts the phosphorylation and nuclear entry of PPAR-gamma. The results suggest that Dab2 is required for the excessive calorie-induced differentiation of an adipocyte progenitor cell population that presents in young and is depleted in older animals.
3.1 Introduction and background

Obesity is defined as accumulation of excessive fat. It is generally believed to relate with many metabolic syndromes including high blood pressure, type 2 diabetes, coronary heart disease, stroke, arthritis, sleep apnea and breathing problems and certain types of cancers (2000). The ultimate imbalance between extra caloric intake and lack of energy expenditure leads to obesity. Animals control the balance of energy intake and expenditure in a highly integrated manner. Excessive energy is mainly stored as lipid components in adipose tissue throughout the body (Stubbs and Tolkamp 2006). Fat cells also called adipocytes are the main storage site of excessive energy. There are two types of adipose tissues with distinct properties, white adipose tissue and brown adipose tissue. White adipose tissue contains a large, single lipid droplet which account for the majority of cell volume, while brown adipocytes have high mitochondrial contents and multi-ocular lipid droplets which is responsible for the heat production (Cannon and Nedergaard 2004).

There are two major ways contributing to obesity. Hypertrophy is increase in the volume of the adipocytes while hyperplasia is the adipocytes increase in number (Jo, Gavrilova et al. 2009). Generally, hypertrophy of adipocytes can be observed in all overweight and obesity individuals. Hyperplasia of adipocytes is relatively rare in the adulthood, with the exception in severely obese individuals. Prolonged period of weight gain can still lead to hyperplasia in the case of severe obesity (Hirsch and Batchelor 1976).

In the adulthood, it is often believed that the total adipocyte number remains relatively constant, and the fat weight changes are mainly due to changes in cell volume. In the early life, adipocyte number is already established (Spalding, Arner et al. 2008). As a result, the childhood obesity may contribute to adipocyte hyperpiesia, which leads to predisposing to
obesity in the adulthood. The turnover of adipocyte in the adulthood is relatively slow but still detectable. By carbon 14 dating, the half-life of human subcutaneous fat cells is about 9.5 years (Arner, Bernard et al. 2011).

The cellular origin of white adipocytes is mesenchymal stem cells which are capable to differentiate into adipocytes, osteocytes, chondrocytes as well as myocytes (Gesta, Tseng et al. 2007). Fat stem cells reside in the stromal vascular fractions, which is a mixture of pre-adipocytes, the endothelial cells, mesenchymal stem cells as well as many types of white blood cells. Subpopulations of stromal vascular cells are able to differentiate into mature adipocytes (Riordan, Ichim et al. 2009). These endothelial cells function as a reservoir to generate new adipocytes as pre-adipocytes. The proliferation and differentiation ability of primary pre-adipocytes is influenced by the age the host. Aging reduced the capacity of proliferation and differentiation into mature adipocytes in primary pre-adipocytes (Djian, Roncari et al. 1983; Sepe, Tchkonia et al. 2011).

Mesenchymal stem cells can differentiate in to mature adipocyte by adipogenesis. Firstly, mesenchymal stem cells determine its cell fate into pre-adipocytes. After several rounds of cell proliferation, pre-adipocytes terminally differentiate into mature adipocytes in the present of environmental clues. During adipogenesis, several key transcriptional factors are activated to initiate an adipogenic transcriptional cascade. During these processes, many signaling pathways such as TGF-beta, WNT and MAP kinase are involved (Tang and Lane 2012). Among these signaling pathways, MAP kinase/Erk pathways have opposite effect upon adipogenesis during different stages. In the early stage of adipogenesis, C/EBP-b is phosphorylation and active by glycogen synthase kinase3b and MAP kinase. Subsequently, C/EBP-b activated the expression of C/EBP-alpha and PPAR-g, the master regulator of
adipogenesis (Tang, Gronborg et al. 2005). However, during terminal differentiation stages, ERK1 phosphorylation leads to PPAR-g phosphorylation in the S-112 site, which leads to its nucleus exportation and degradation (Hu, Kim et al. 1996, Adams, Reginato et al. 1997). Thus, MAP kinase inhibits adipocyte differentiation in the late stages.

In this study, we have found that Dab2 conditional deficient mice are resistant to a weight gain induced by the high fat diet. When the mice are fed with a high fat diet, body fat content is significantly lower in Dab2 deficient mice compared with wild type controls, while lean tissue mass remains similar. We found that adipocyte number but not size is reduced in Dab2 deficient mice. Dab2 protein is highly expressed in white adipose tissues. We have found that Dab2 protein is increased greatly during adipogenic differentiation of both NIH 3T3-L1 and mouse embryonic fibroblast (MEF) cells. However, Dab2 deficient MEF cells are impaired in adipogenic differentiation. The absent of Dab2 allows an increased nuclear localization of protein kinase ERK and the activated MAPK inhibits peroxisome proliferator- activated receptor (PPAR) gamma. These data suggest Dab2 plays a role in a signaling pathway controlling adipocyte differentiation

3.2 Dab2 conditional deficient mice are resistant to high fat diet-induced weight gain

We were able to bypass the requirement of Dab2 in early embryogenesis and produced Dab2 conditional knockout mice with an essentially complete absence of Dab2 (Moore et al., 2013), which allow us to investigate the physiological roles of Dab2 in intact animals. The Dab2 null mice appear largely normal, though we observed a small increase in serum cholesterol (Moore et al., 2013), which is consistent with a role of Dab2 as an endocytic adaptor for the LDL receptor. To investigate further the importance of Dab2 in LDL metabolism, we challenged the Dab2 null (dab2 (f/df):Sox2-Cre) mice with high fat diet
Unexpectedly, we observed a profound resistance in HFD-induced weight gain in Dab2-deficient mice. We documented the weight gain of Dab2 null and control mice on normal or high fat chow over a 6-month period, with weekly record weight for each animal (Figure 1). A weight difference was observed in both male and female mice. Since there were large differences between the sexes, we used only male mice in subsequent analyses. Also, dab2 heterozygous littermates were used as controls for comparison with the Dab2 null mice, since previously we observed that dab2 heterozygous mice were identical to wildtype, and our breeding produced few wildtype (dab2 (+/f)) littermates. On normal chow, the weight difference between Dab2 null and sufficient mice was small over a 28 week period (Figure 13A). The most dramatic difference in diet-induced weight gain between Dab2 conditional knockout (CKO) and heterozygous (HET) mice was at around 3-4 months, when the Dab2 Het mice had approximately 30% weight than Dab2 null mice. The difference was still substantial at the end of the experiment, of around 20% difference (Figure 13A). The groups of wildtype mice were indistinguishable in weight gain as the HET mice, as shown by the time point at 28 weeks (Figure 13A). Using Pixi-densitometer to analyze body composition, we found that the different fat mass accounted for the differential weight gain, while the lean mass was essentially the same between Dab2 CKO and HET mice (Figure 13B, C). The fat mass of Dab2 HET was about double of the CKO mice, and the fat mass was about 39% and 22% of body weight, respectively. The reduced fat deposition was very obvious upon dissection (Figure 13 D, E). The white adipose tissues from all fat depots were reduced in Dab2 null mice: inguinal, 63%; subcutaneous, 76%; gonadal, 64%; and mesenteric, 71%, compared to those of HET mice (Figure 13F). No
Figure 13. Dab2 conditional knockout mice are resistant to high fat diet-induced weight gain (A) Groups of wildtype (WT), Dab2 conditional knockout (dab f/f:Sox2-cre) (CKO), and heterozygous (HET) controls (both dab f/f and dab +/f:Sox2-cre) male mice at 7 weeks of age were placed on either normal chow (NC) or high fat diet (HFD) for an additional 28 weeks (6 and a half months). Weight was determined weekly, and the values of individuals in each group (10 to 11 animals) were averaged and shown with standard deviations. The weight for the WT group (n=7) on high fat diet is shown for only the last time point. (B) The fat and lean tissues of Dab2 CKO and HET litter mates of 6 months of age that were fed a high fat diet were determined using dual-X-ray absorptiometry. Representative PIXI images of a HET (dab2 +/f:Sox2-cre) and a CKO (dab2 f/f:Sox2-cre) are shown. (C) The lean, fat, and total body mass were determined by the DEXA system and the means and standard deviations from a group of 11 HET and 8 CKO mice were presented. The percentage of fat mass of the body weight was calculated. (D) Representative examples of the exposed peritoneal fat tissues comparing a dab2 CKO and a HET litter mate after on HFD for 6 months. (E) The representative dissected gonadal fat tissues are shown, and (F) the quantitative measurement of dissected fat tissues (inguinal, brown, subcutaneous, gonadal, and mesenteric) were determined in 6 each of the HET and CKO male mice.
change occurred in brown fat mass. These data indicate that Dab2 deficiency leads to resistance to caloric-induced adipose tissue expansion and weight gain.

3.3 Adipose cell number rather than cell size is affected by Dab2 deficiency

Despite the striking lean phenotype of the Dab2 null mice fed a high fat diet, serum lipid profiles showed only subtle differences between the CKO and HET mice (Figure 14A). Glucose and cholesterol levels had small but statistically significant increase, though serum triglycerides were unchanged. HFD increased the cell size of the adipocytes in both CKO and HET mice upon histological analyses (Figure 14B). Surprisingly, Dab2 null adipocytes were even slightly larger, rather than reduced size compared to those of wildtype (Figure 14A), as confirmed by computer assisted image analysis (Figure 14C). Dab2 is abundant in adipose tissues shown by Western blot (Figure 14D), and this was also confirmed by immunofluorescence microscopy analysis of gonadal adipose, where Dab2 staining was dotted in the narrow cytosolic space located between the perilipin-surrounded lipid droplets (Figure 14E). Cell number of the adipose tissues was also quantified by determining the genomic DNA content. In an equivalent weight of fat tissue, the DNA content was equal between Dab2 CKO and HET. From these data, we conclude that the absence of Dab2 in adipocytes does not affect the cell size but rather affects the total cell number, and Dab2 CKO mice on high fat diet have a 30-40% reduction in fat cell number compared to HET mice.

3.4 Age and adipocyte restricted Dab2 deficiency on high fat diet-induced weight gain

The dab2 CKO mice had been produced and studied for several years already. The mutant mice seemed exceedingly normal, and a lean phenotype was only observed when
**Figure 14. Characterization and analysis of adipose tissues and cells from Dab2 deficient mice**

(A) Blood chemistry analysis was performed on Dab2 CKO and HET mice that were fed with a HFD. BUN, Blood Urea Nitrogen; Crea, creatinine; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

(B) The histology of gonadal adipose tissues is shown for Dab2 CKO and HET controls in both normal chow and high fat diet (HFD).

(C) The images were analyzed by computer program assisted image analysis (MetaMorph) for the diameter of the cells as an indication of cell size. The cell size distribution is shown as percentage of cells at each increment of diameter. HFD induced a similar increase of adipocyte cell size in both Dab2 knockout and heterozygous mice.

(D) Dab2 protein expression was determined by Western blot in protein extracts from various tissues dissected from the mice. Dab2 proteins were completely absent in tissues from the Dab2 CKO mice.

(E) Analysis of Dab2 and perilipin-1 in gonadal fat tissues from dab2 CKO and HET mice using immunofluorescence microscopy.
the mice were fed a high caloric diet. Typically, we placed young mice on a designated
diet (either high fat or normal) once the pups were genotyped and weaned, at around 3-4
weeks of age. We also tested the impact of a high fat diet on older mice (6 months). In
the experiments, the littermates were first kept on regular chow (normal chow) over a 6-
month period, and were started on a high fat diet for another 3 months (11 weeks) (Figure
15A). Interestingly, we observed no difference in weight gain between the CKO and
HET mice (Figure 15A). In numerous tests in addition to the specific experiment shown
in Figure 1A, we have consistently observed the lean phenotype of the Dab2 CKO mice
on a high fat diet starting at 3 weeks to two months of age, but no impact after 6 months
of age. Thus, it appears that the effect of Dab2 on a high fat diet-induced weight gain is
present only in young mice, and the impact is lost in older mice. We also examined if the
effect of Dab2 is adipose specific by testing in mice with dab2 deletion restricted to
adipose tissues using an Ap2-cre line (He et al., 2003). By crossing the dab2 f/f line with
an Ap2-cre line, we generated conditional knockout (dab2 (f/df):Ap2-cre) and
heterozygous control (dab2 (+/f):Ap2-cre and dab2 (f/df)) mice. The adipose-restricted
dab2 CKO mice also showed a reduced weight gain compared to HET after being placed
on a high fat diet (Figure 15B). The weight of dab2 (f/df):Ap2-cre CKO was 84% for
females, and 87% for males of the HET mice, compared to 76% for the dab2 (f/df):Sox2-
cre male mice (Figure 15B). Thus, dab2 deletion within the adipocyte lineage affected
HFD-induced weight gain, though the magnitude was not as large as in mice with
complete dab2 deletion.
Figure 15. Adipocyte restricted Dab2 deficiency and stating age on high fat diet-induced weight gain (A) Weights were determined weekly for groups of Dab2 CKO and HET mice that were initially fed a normal chow and then switched to a HFD at 6 months of age. (B) dab2 (f/df) mice were crossed with Fabp4-cre mice to produce mice in which Dab2 is deleted only in adipocyte tissues (dab2 (f/df):Fabp4-cre). These adipocyte tissue-specific Dab2 deficient mice were tested on a high fat diet starting at 7 weeks of age for an additional 28 weeks. The weights were determined and compared with control littermates (dab2 (f/df) and dab2 (f/df):Fabp4-cre).
3.5 **Dab2 is required for efficient adipocyte differentiation in cultures**

The facts that Dab2 is expressed in adipocytes, and that Ap2-cre lineage restricted dab2 deletion also show a reduced weight gain, suggest an adipocyte cell autonomous function of Dab2. Thus, we investigated the effect of dab2 deletion on adipocyte differentiation in cultured cells. Multiple mouse embryonic fibroblast (MEF) lines were prepared from the embryos generated from crosses between male dab2 (+/df):Sox2-cre and female dab2 (f/df) mice. The cells were expanded, genotyped, and assayed for in vitro adipocyte differentiation. In all the dab2 CKO and HET MEF lines, a consistent reduction in the degree of adipose differentiation was observed in CKO compared to HET lines based on obvious morphological changes (Figure 16A), and staining of adipocytes with Oil-O Red (Figure 16B, C). Quantitation indicated that the lipogenic differentiation of the dab2 CKO cells was reduced to about 40% of HET cells (Figure 16D). RT-PCR analysis of mRNA extracted from the MEFs before and after differentiation showed that the induced expression of adipogenic markers were remarkably reduced in Dab2 CKO compared to HET MEFs: C/EBPα, 43%; Leptin, 58%; PPARγ1, 53%; and PPARγ2, 43%. In addition, we found that Dab2-deficient embryonic stem (ES) cells and mesenchymal stem cells had a similar reduction in lipogenic differentiation compared to HET control cells in respective differentiation conditions. Multiple ES lines and mesenchymal stem cells preparations isolated from the mutant mice were tested to produce consistent results. Furthermore, Dab2 suppression using siRNA in the 3T3L1 adipocyte differentiation assay also indicated a reduction of adipogenic activity to 20-30%. Thus, consistent results generated from several cell types, and suppression of Dab2 by gene knockout or
Figure 16. Dab2-deficient mouse embryonic fibroblasts (MEFs) have reduced capacity for adipocyte differentiation

(A) dab2 HET and CKO MEFs were used for differentiation into adipocytes following incubation for two days in “Induction Medium” and then culturing in “Differentiation Medium” for 4 to 14 days. Morphology of the cells is shown for Day 0, 2, and 4 in “Differentiation Medium”. (B) Following differentiation for 7 days in the “Differentiation Medium”, the plates were stained with Oil-O Red. Two clones each of Dab2-deficient and -positive (HET) MEF cells are shown. (C) Representative magnified images of the cells stained with Oil-O Red under a microscope are shown. (D) The amount of Oil-O Red was quantified, and triplicate assays from two independent clones are shown for each genotype. (E) The cells were analyzed for expression of adipocyte genes using qRT-PCR, comparing untreated to 7-day differentiated MEFs.
siRNA suppression, confirmed a cell autonomous role of Dab2 in promoting adipocyte differentiation.

3.6 Dab2 expression is induced in adipocyte differentiation, correlating with suppression of phospho-MAPK

The impacts of Dab2 on signaling pathways during adipogenic differentiation were investigated. In multiple experiments, we observed the robust induction of Dab2 expression as HET and wildtype cells were progressively differentiated, accompanying the increasing PPARγ and C/EBPα (Figure 17A). In comparison, the induction of PPARγ and C/EBPα was blunted in the dab2 CKO cells. Adipocyte differentiation accompanied growth suppression as indicated by the reduction in the proliferation indicator PCNA, but the PCNA reduction was not observed in dab2 CKO cells. In Dab2 deficient cells, phospho-Erk1/2 was elevated, but total Erk1/2, Grb2, Sos and beta-actin were not altered (Figure 17A). Increased Erk1/2 activation and reduced PPARγ were also demonstrated in another experiment where cells were fractionated into cytoplasmic (Grb2 as a marker) and nuclear (lamin A/C as a marker) protein extracts (Figure 17B). On day 4 and 9 of differentiation, phospho-Erk was reduced in HET cells, but remained high in dab2 CKO cells. In correlation, PPARγ was reduced in the dab2 CKO cells, especially in the nuclear fraction (Figure 17B). The markers were also examined analyzed in individual cells by immunofluorescence microscopy. At day 4, the differentiating adipocytes were identified using Bodipy staining for the intracellular lipid droplets and were also positive for Dab2 (Figure 17C). In these Bodipy-positive cells, PPARγ showed positive nuclear staining. In comparison, Bodip-i-positive cells were few and PPARγ appeared cytoplasmic for the dab2 CKO cells (Figure 17C). In the case of C/EBPα, dab2-null cells had fewer Bodipy
Figure 17. Correlation between Dab2, PPARγ, and C/EBPα in adipocyte differentiation of mouse embryonic fibroblasts

Dab2 HET and CKO MEFs were undergoing differentiation into adipocytes by 2 days in the “Induction Medium” followed by a time course in the “Differentiation Medium”. (A) Total cell lysates were analyzed by Western Blot. (B) The cells at day 1, 4, and 9 of culture in “Differentiation Medium” were fractionated into cytoplasmic and nuclear extracts. The proteins were analyzed for the presence of PPARγ and phospho-Erk in each fraction by Western blot. Grb2 was used as a cytoplasmic marker, and lamin A/C was used as a nuclear marker. (C) Representative examples of immunofluorescence detection of PPARγ, Dab2, and Bodipy are shown. A quantitation indicates that PPARγ expression and nuclear localization, Dab2 expression, Bodipy positivity in the same cell correlate in the Dab2 HET cell population. More than 200 cells from 5 images acquired for each sample were quantitated to produce the statistics.
and C/EBPα-positive cells, but C/EBPα remained nuclear (Figure 17D). On day 2 of differentiation when Dab2 expression was induced and Erk1/2 activation had started to be suppressed, a clear correlation between Dab2, phospho-Erk, and PPARγ expression was observed (Figure 18). Within the cell population, Dab2 and phospho-Erk1/2 showed a mutually exclusive expression pattern: when Dab2 was induced, phospho-Erk1/2 was negative, as quantified in over 400 cells examined (Figure 18A). The cells were positive for Dab2 in 14%; positive for phospho-Erk1/2 in 16%, and only 1.6% of the cells were positive for both Dab2 and phospho-Erk1/2. Similarly, nuclear PPARγ and phospho-Erk1/2 were also mutually exclusive (Figure 18B), with only about 1% of the cells were positive for both phospho-Erk1/2 and PPARγ. As expected, Dab2 expression and PPARγ nuclear positivity were correlative, with more than 90% of the cells expressing both Dab2 and nuclear PPARγ (Figure 18C). These results demonstrate a close association between Dab2 induction, phospho-Erk1/2 suppression, and PPARγ nuclear expression. The data suggested the idea that elevated Erk1/2 activation in dab2 CKO cells leads to nuclear exclusion of PPARγ and a reduction in adipogenic differentiation.
Figure 18. Regulation of P-Erk by Dab2 in adipocyte differentiation. Dab2 HET and CKO MEFs were differentiated into adipocyte. (A) Representative examples of immunofluorescence of p-Erk1/2 and Dab2 are shown. A quantitation indicates that P-Erk positivity and Dab2 expression are inversely correlated. (B) Representative examples of immunofluorescence detection of P-Erk and adipocytes are shown. A quantitation indicates that P-Erk expression and PPARγ are inversely correlated. More than 200 cells from 5 images acquired for each sample were quantitated to produce the statistics. More than 200 cells from 5 images acquired for each sample were quantitated to produce the statistics.
3.7 Regulation of Erks activation by Dab2 through modulation of Sos-Grb2 association

A potential mechanism of Dab2 on the activity of the ras-Erk1/2 pathway is its binding to Grb2 in competition with Sos (Xu et al., 1998). We first assessed the impact of Dab2 on the association of Grb2 and Sos in the process of adipocyte differentiation of MEFs using co-immunoprecipitation. In the total cell lysate, induction of Dab2 in HET cells reduced phospho-Erk in CKO cells, and Sos, Grb2, and total Erk remained unchanged (Figure 19A). Immunoprecipitation of Grb2 was used to determine the association with Dab2 and Sos (Figure 19B). Western blot analysis of Dab2 and Sos in the immunoprecipitation of Grb2 indicated that on day 1 of differentiation, Sos, but not Dab2, was associated with Grb2 in HET cells. However, on Day 4 and 9 of differentiation, Dab2 replaced Sos in the Grb2 immunoprecipitation (Figure 19B). In the absence of Dab2, Sos level was maintained the same amount in Grb2 immunoprecipitation. The results are consistent with the idea that Dab2 increases upon adipocyte differentiation, competes with Sos for binding to Grb2, leads to the dissociation of Sos from Grb2, and hence a reduced Erk phosphorylation/activation.
Figure 19. Regulation of Erk activation by Dab2 through modulation of Sos-Grb2 association Dab2 HET and CKO MEFs were undergoing differentiation into adipocyte. (A) Immunoprecipitation of Grb2 in Dab2 HET and CKO MEF cells during 1, 4, 9 days of adipocyte differentiation. Western blot analysis of Dab2, p-Erk1/2, Sos1, and Grb2 was performed on total cell lysates and Grb2 co-immunoprecipitation (co-IP) samples. (B) Immunofluorescence images of Sos1 and Grb2 in Dab2-positive and null cells.
3.8 Inhibition of Erk activation partially restores adipose differentiation of Dab2 null cells

Since the above data suggest the main effect of Dab2 is on suppressing Erk activation, we tested the impact of Erk inhibition on adipocyte differentiation in Dab2 null MEF cells. Inclusion of the MEK inhibitor U0216 for 4 days partially restored the degree of adipocyte differentiation of the dab2 CKO cells (Figure 20A). In the presence of the MEK inhibitor, differentiation of Dab2 null MEF cells reached the same level as that of HET cells, though the dab2 HET cells achieved higher degree of adipocyte differentiation in the presence of the inhibitor. Western blot analysis of markers indicated U0216 suppressed phospho-Erk and increased PPARγ in the Dab2 null cells (Figure 20B). Thus, the results support the conclusion that Dab2 regulates adipocyte differentiation by suppressing Erk activation, which then phosphorylate and down regulate PPARγ.
Figure 20. MEK inhibitor U0216 can partially restore adipose differentiation of Dab2 null MEF cells (A) Dab2 HET and CKO MEFs were first treated with “Induction Medium” for 2 days and then were cultured in the “Differentiation Medium” to mature into adipocyte in the presence or absence of MEK inhibitor U0216 for 4 days. Morphology of the cells is shown. (B) The cells were analyzed using Western blot for markers including Dab2, phospho-Erk, PPARγ, C/EBPα, and beta-actin.
3.9 Summary and significance

In the analysis of conditional knockout mice for Dab2, an endocytic adaptor protein and a signal transduction regulator, we uncovered a role of Dab2 in modulation of adipocyte differentiation, as reported in this study. Based on analysis of the mutant cells, we proposed a model in which Dab2 mediates nutrition signal to modulate Ras/MAPK pathway and thus impacts on the differentiation and recruitment of new adipocytes into adipose tissues (Figure 21). According to this model, Dab2 expression is inducible by excessive calorie. In normal condition, Ras/Erk activity suppresses PPARγ and thus lipogenic differentiation. The induction of Dab2 leads to the suppression of Ras/Erk1/2 activity through Dab2 binding and sequestration of Grb2 from Grb2/Sos complex, which results in the reduction of Ras/Erk activity. Consequently, PPARγ is allowed to enter the nucleus to initiate transcription that promotes lipogenic differentiation.
Figure 21. Schematic illustration of Dab2 in regulation of adipocyte differentiation

The activity of the Ras/Erk pathway suppresses adipocyte differentiation by phosphorylation of PPARγ, which leads to its nuclear export and degradation. Our results suggest a model where excessive calorie induces expression of Dab2 in adipocyte precursors. The increased Dab2 binds Grb2 and competes with Sos1, leading to the dissociation of Sos1-Grb2 complex that is recruited to a receptor tyrosine kinase (RTK) and consequently the reduced Erk activity. The absence of Erk activation allows nuclear accumulation of PPARγ, induction of C/EBPα, and together these transcription factors initiate gene expression that promotes adipocyte differentiation. In the Dab2 null cells, Erk activation remains elevated and subsequently PPARγ is excluded from the nucleus and undergoes degradation, leading to reduced adipocyte differentiation.
Chapter IV: PHYSIOLOGICAL ROLE OF DAB2 IN THE ENDOCYTOSIS OF LIPOPROTEIN AND CHOLESTEROL METABOLISM

Clathrin mediated endocytosis of LDL receptor required cargo specific adaptor proteins. PTB domain containing protein such as Dab2 and ARH can preferentially interact NPXY motif and facilitate the endocytosis of LDL receptor. Since Dab2 and ARH have homologous function in LDLR endocytosis, ARH is primarily expressed in the liver and Dab2 is ubiquitously synthesis through the body, we hypothesis that Dab2 plays a compensatory but not redundant role in LDL endocytosis in the peripheral tissues while ARH is mainly responsible for those in the liver. In this study, we have generated compound knockout mice for both Dab2 and ARH. The double knockout mice are viable and grossly normal. When these mice are challenged with high sucrose diet, we found that double knockout mice have a synergistic increase in cholesterol and LDL levels comparable to LDL receptor alone. Surprisingly, the double knockout mice also develop hepatic steatosis when fed with a high sucrose diet. In the mouse embryonic fibroblasts, we found that in the ARH-/- background, Dab2 depletion can greatly reduce LDL endocytosis activity, almost to the level of LDLR-/-.

The LDLR protein level but not mRNA level is reduced in Dab2 and ARH double knockout MEF cells.
4.1 Introduction and background

Cholesterol is a sterol lipid which is an essential component of animal cell membrane as well as a precursor for the steroid hormones, bile acid and vitamin D. (Hanukoglu 1992) Cholesterol is mainly synthesized in the liver and delivered to other cells. Since cholesterol is almost insoluble in the serum, it is transported in the circulation system as particles that have amphiphilic lipoproteins and lipids with outward facing surface water soluble and inward facing surface lipid soluble. (Olson 1998)

Circulating LDL particles are the main carrier transport the cholesterol from liver, through blood stream to the other cells in all parts of the body. (Hevonoja, Pentikainen et al. 2000) Each LDL particle carry about 1000 molecules of cholesteryl ester and 500 free cholesterol molecules. (Yang, Gu et al. 1989) LDL particles is consist of a hydrophilic surface layer consist of apoB-100 protein and a hydrophobic core of cholesterol and cholesterol ester and triglyceride. (Hevonoja, Pentikainen et al. 2000) In the physiological state, normal cholesterol delivery by LDL particle is required for the maintenance of normal cell membrane rigidly and production of certain types of hormones. However, in the pathological state, atherosclerosis is caused by excessive cholesterol accumulate in artery walls. The source of cholesterol is plasma lipoprotein, particularly LDL. (Waters and Pedersen 1996)

The major pathway for removal the LDL particle from the plasma is facilitated by the receptors mediated endocytosis on the cell surface membrane in contact directly with serum. The amphiphilic lipoproteins apoB-100 mediates the binding of LDL particles to LDL receptors. (Jeon and Blacklow 2005) The LDL receptor can recognize its ligand apoB-100 on the LDL particle surface. Upon binding of LDL particles, LDL receptors cluster in to
clathrin-coated pits. The invagination of coated pits form coated vesicles, which latter converted to the endosome. In the late endosome, the PH dependent conformational change causes the dissociation between LDL particles and LDL receptors. LDL particles are sorted into lysosome and degraded releasing free cholesterol. Meanwhile, LDL receptors are recycled back to cell surface membranes. (Schneider and Nimpf 2003)

Clathrin mediated endocytosis of LDL receptor required cargo specific adaptor proteins which can recognize LDL receptors and then recruit them in clathrin coated endocytic pits. (Traub 2009) The FxNPxY motif on the cytoplasmic tail of LDL receptor can be specifically recognized by two endocytic adaptor protein Dab2 and autosomal recessive hypercholesterolemia (ARH). (Maurer and Cooper 2006) In human, ARH gene mutation can cause elevated circulation LDL and cholesterol level as well as impaired LDL endocytosis. (Garcia, Wilund et al. 2001, Jones, Hammer et al. 2003) In Arh knockout mouse model, when mice are fed with high sucrose diet, serum cholesterol level in Arh−/− mice were higher than wild-type, but much lower than those in LDL receptor null mice. (Jones, Garuti et al. 2007) The skin fibroblasts from ARH patients can take up LDL normally even with ARH mutation, Dab2 acts as an adaptor for LDL receptor independent of ARH. (Eden, Sun et al. 2007) In HeLa cell, LDLR endocytosis is partially but significantly disrupted by Dab2 depletion. However, when both Dab2 and ARH are suppressed, LDLR endocytosis is strongly inhibited to the similar degree as clathrin depletion. (Maurer and Cooper 2006) These data suggest Dab2 and ARH play similar but not redundant roles as endocytic adapters for LDL receptor endocytosis.

The Brown and Goldstein laboratory have found mutations in the cytosolic domain of LDL receptor prevent the endocytosis of LDLR. Further mapping analysis have demonstrate that
FxNPxY motif which can interact with the PTB domain is required and sufficient for LDLR internalization. It is known that PTB domain such as Dab1, Dab2, Numb and ARH can preferentially interact with NPXY motif in the non-phosphorylated state. The PTB domain is also able to interact with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) via distinct site from the NPXY motif binding site. (Yun, Keshvara et al. 2003)

Since Dab2 and ARH have homologous function in LDLR endocytosis, ARH is primarily expressed in the liver and Dab2 is ubiquitously synthesis through the body, we hypothesis Dab2 plays a compensatory but not redundant role in LDL endocytosis in the peripheral tissues while ARH is mainly responsible for those in the liver. In this study, we have generated compound knockout mice for both Dab2 and ARH. The double knockout mice are viable and grossly normal. When these mice are challenged with high sucrose diet, we found that double knockout mice have a synergistic increase in cholesterol and LDL levels comparable to LDL receptor alone. Surprisingly, the double knockout mice also develop hepatic steatosis when fed with a high sucrose diet. In the mouse embryonic fibroblasts, we found that in the ARH-/- background, Dab2 depletion can greatly reduce LDL endocytosis activity, almost to the level of LDLR-/- . The LDLR protein level but not mRNA level is reduced in Dab2 and ARH double knockout MEF cells.
4.2 Differential cellular expression of Dab2, ARH and NUMB in primary cells

To investigate the biological functions of Dab2 and its close homologues ARH and Numb, we have characterized the expression of Dab2 ARH and Numb in primary cell cultures harvested from Dab2 conditional knockout (Dab2 f/f:Sox2-cre) mice. In the primary mouse embryonic fibroblast (MEF) cell, we have found Dab2, Numb and ARH are all expressed in MEF cells by Western blots. (Figure 22 A) By immunofluorescence staining, NUMB and Dab2 both co-localized with adaptor protein AP2. (Figure 22 B) In the mouse Embryonic Stem (ES) cells can be forced to differentiate into primitive endoderm lineage by all-trans Retinoid Acid (RA). The primitive endoderm marker Dab2 can be induced by RA in ES cells. Similar to Dab2, Numb and ARH both are induced by retinoid acid in ES cells. (Figure 22 C) In additional to that, Dab2 Numb and ARH are expressed in Mouse Ovarian Surface Epithelial (MOSE) cells and Mouse Mammary Gland Epithelial (MMGE) cells. Noticeably, NUMB and ARH protein level compensate for Dab2 loss in mammary epithelial cells. (Figure 22 D) These data indicated that Dab2 and its close homologues ARH and Numb have similar expression pattern. Since the functional similarities amount those endocytic adaptors, expression of ARH and numb may partially explained the lack of adult phenotypes in Dab2 deficient mice.
Figure 22. Differential cellular expression of Dab2, ARH and NUMB in primary cells (A) Western blot (B) and Immunofluorescence staining of Dab2, Numb and ARH in the mouse embryonic fibroblast (MEF) cells. (C) Western blot analysis of Dab2 Numb and ARH in ES cells treated with retinoid acid. (C) Western blot analysis Dab2 Numb and ARH in Mouse Ovarian Surface Epithelial (MOSE) cells and Mouse Mammary Gland Epithelial (MMGE) cells
4.3 Generating Dab2 and ARH double knockout mice

To investigate the compensatory roles of Dab2 and ARH in vivo, we have mate conditional knockout (Dab2 f/f:Sox2-cre) in to ARH-/- background. The strategy for generating Dab2 and ARH double knockout mice is illustrated in Figure 22. The ARH mutant mice were originally generated from Herz lab. (Jones, Hammer et al. 2003) In original paper, the screening primers were based on the neomycin resistant cassette which flanked by two loxP sites (floxed), in the present of Cre recombinase, the neomycin resistance gene was cut and removed (deltafloxed). Since the primers described in the original primers were not able to detect the deltafloxed alleles, we developed a novel set of primers to genotype the ARH mutant mice. Using 4 PCR primers at 1uM each, we can genotype gDNA and distinguish the 3 different alleles (wild type, deltaflox & KO) by size of PCR amplicon. (Figure 22B and C) Since during early embryonic development, ARH expression was detected in the parietal endoderm, we suspected that Dab2 and ARH double knockout mice may be embryonic lethal.(Maurer and Cooper 2005) However, Dab2 and ARH double knockout mice are viable and grossly normal, which make it possible to characterize the adult phenotypes of Dab2 and ARH double knockout mice.
Figure 23. Generating Dab2 and ARH double knockout mice. (A) Mating strategy for generating Dab2 and ARH double knockout mice. (B) Schematic illustration of ARH gene deletion in mutant mice. The genotyping primers are indicated as illustration. (C) Examples of PCR screening assay for ARH mutant mice, 3 different alleles (wild type, deltaflox & KO) are distinguished by size of PCR amplicons.
4.4 Synergistic Increase in Cholesterol and LDL level in double knockout mice

In Arh knockout mouse model, when mice are fed with high sucrose diet, serum cholesterol level in Arh+/− mice were higher than wild-type, but still much lower than those in LDL receptor null mice. (Jones, Garuti et al. 2007) These data suggested LDL receptor endocytosis may be facilitated by other endocytic adaptors other than ARH. We hypothesized that Dab2 may also involve in the process LDL endocytosis in vivo. To reveal the possible serum cholesterol phenotype in Dab2 and ARH double knockout mice, we challenged the mice with high sucrose diet as described in the literatures (Jones, Garuti et al. 2007). After 3 month on high sucrose diet, mice were sacrificed to collect serum and tissues. Serum samples were analyzed for blood chemistry. Serum cholesterol and LDL level was slightly elevated Dab2 deficient mice compared with wild type mouse. Serum VLDL or TG levels are similar between these two groups. (Figure 23A)

However, in the ARH knockout background, the total serum cholesterol and LDL level are elevated in Dab2 and ARH compound knockout mice compared with ARH knockout mice alone. (Figure23A) Meanwhile, serum VLDL and TG levels were also slightly elevated in compound knockout mice than ARH knockout alone. These data indicated that Dab2 and ARH both participate in LDL endocytosis. Dab2 deletion alone is not sufficient to induce a significant elevation in serum cholesterol and LDL level since ARH also play a major role in LDL endocytosis. The Dab2 and ARH compound knockout mice have similar serum cholesterol and LDL levels to the LDL receptor knockout mice. These data suggested Dab2 plays a compensatory but not redundant role in LDL receptor endocytosis.
Figure 24. Blood chemistry of Dab2 and ARH double knockout mice and LDLR knockout mice. (A) Serum cholesterol and low density lipoprotein (LDL), triglycerides, high density lipoprotein (HDL) and very low density lipoprotein (VLDL) levels in Dab2 and ARH double deficient mice and LDLR knockout mice (B) Serum samples of Dab2 and ARH double knockout mice and LDLR knockout mice after centrifugation at 4°C.
4.5 Impaired LDLR endocytosis in double knockout MEF cells

To further assay the LDL-receptor endocytosis activities, we have harvested mouse embryonic fibroblast (MEF) cells from wild type, Dab2 -/-, ARH -/-, Dab2-/- ARH-/- and LDLR -/- mice. Bodipy-labeled LDL particles were incubated with MEF cells to assay the LDL-receptor endocytosis activities. After incubation, green fluorescence positive cells were imaged and quantified (Figure 23A and B). The number of green fluorescence positive MEF cells from different genotypes was compared. Among different genotypes, wild type MEF cells have the most green fluorescence positive cells. Dab2 knockout MEF cells have significantly less green fluorescence positive cells than wild types. Similar to LDLR knockout cells, Dab2 and ARH double knockout MEF cells have much less green fluorescence positive cells than ARH knockout MEF. These data indicated that in the ARH-/- background, Dab2 depletion can almost deplete LDLR endocytosis activity. The LDLR knockout MEF cells and compound knockout MEF cells both have very low LDLR endocytosis activity, suggesting that the endocytosis of LDL receptors are depended on endocytic adaptors Dab2 and ARH. From \textit{in vitro} cell culture data, deletion of both Dab2 and ARH can almost abolish the endocytosis of LDL receptors to the similar levels of the LDLR knockout MEF cells.
Figure 25. LDL update assay in MEF cells: Bodipy- labeled LDL particles were incubated with wild type, Dab2 -/-, ARH -/-, Dab2-/- ARH-/- and LDLR -/- MEF. Green fluorescence positive cells were imaged (A) and quantified in (B).
4.6 Liver phenotype in the double knockout mice

In the literatures, ARH-/- mice are grossly and no obvious adult phenotypes. (Jones, Hammer et al. 2003) To test the possibilities that deletion of both endocytic adaptors Dab2 and ARH may change the tissue morphology, we analyzed the Dab2 and ARH double knockout mice on high sucrose diet by histological sections. We have found no obvious phenotypical abnormalities in the kidneys sections or other tissues expressing Dab2 and ARH. (Figure 25A) However, with H&E staining and electron microscopy, we found that in the double knockout liver, the clear spaces between nucleus and cytoplasm were greater with large intracellular vesicles, which indicating hepatic steatosis in the Dab2 and ARH double knockout mice as well as in LDLR-/- mice. (Figure 25A and B) By staining lipid droplet with BODIP dye, the intracellular vesicles found in compound knockout mice are positive for green fluorescence, indicating that these vesicles are composed of neutral lipids. Dab2 is absent hepatocytes, although its expression is detected in nonparenchymal fraction (Keyel, Mishra et al. 2006). By immunofluorescence staining, we found that Dab2 expression is absent in the parenchymal cells but present in the sinusoidal endothelial cells in liver sections. (Figure 25B) In summary, ARH’s expression is found in parenchymal cells while Dab2’s expression is detected in nonparenchymal cells. The Dab2 and ARH double knockout mice develop liver steatosis when fed with a high sucrose diet.
Figure 26. Liver phenotypes of Dab2 and ARH double deficient mice. A. H&E sections of liver and kidney from Dab2 ARH double deficient mice. Electron microscopy image of liver sections from Dab2 ARH double deficient mice. B. Immunofluorescence staining of Dab2 and lipid droplet with BODIP dye.
4.7 LDLR protein level but not mRNA level is reduced in Dab2 and ARH double knockout MEF cells

In the skin fibroblasts from ARH mutant patients, Dab2 depletion profoundly reduced LDL-receptor activity because of a great reduction in LDL-receptor protein, but not mRNA, suggesting a novel role for Dab2 in normal translation of LDL receptor mRNA in ARH -/- fibroblasts. (Eden, Sun et al. 2007) With the MEF cells from wild type, Dab2 -/-, ARH -/-, Dab2-/- ARH-/- and LDLR -/- mice, we assay the protein and mRNA levels of LDLR in these MEF cells. LDLR protein levels were reduced in the Dab2 and ARH double knockout cells. (Figure 26A) However, the mRNA levels are similar in Dab2 and ARH double knockout MEF cells to the other genotypes of MEF cells. These data indicated deletion of both Dab2 and ARH can affect the translation of LDL receptor mRNA.

4.8 Summary and significance

In this study, we have characterized cellular expression of Dab2, ARH and NUMB in primary cells. We successfully generated viable Dab2-/- and ARh-/- double deficient mice. In these double knockout mice, we observed an increase in serum LDL and cholesterol levels and hepatic steatosis. With the MEF cells generated from those mice, we found that in the ARH-/- background, Dab2 depletion can further impair LDLR endocytosis activity by reduced in protein but not mRNA level.
Figure 27. LDLR protein level but not mRNA level is reduced in Dab2 and ARH double knockout MEF cells: (A) Western blot analysis of LDLR, ARH, Dab2 and Actin protein levels in Dab2 -/-, ARH -/-, Dab2-/- ARH-/- and LDLR -/- MEF cells. (B) Quantitative real-time RT-PCT analysis of LDLR and VLDLR mRNA level in Dab2 -/-, ARH -/-, Dab2-/- ARH-/- and LDLR -/- MEF cells.
CHAPTER V: MATERIALS AND METHODS

Treatment of cells with TGF-beta

Recombinant mouse TGF-beta 1 was purchased from R&D system (7666-MB-005). Recombinant protein powder was resuspended in 1% BSA in PBS. Prior to use in experiments, the latent TGF-beta was activated by acid according to the manufacturer’s protocol. Dosages of TGF-beta were tested and a final concentration of 10 ng/ml was used to treat mammary epithelial cells.

Cell growth assay

Cell growth assays were performed using the cell proliferation reagent WST-1 (Roche Cat. No. 11 644 807 001). Cells were seeded at a density of 1,000 cells/ well in 96-well plates in 100 μl of growth media. WST-1 reagent (10 μl) was added to each well in the growth media and incubated at 37oC for 1 hour. Subsequently, a colorimetric assay was performed with a scanning multi-well spectrophotometer at 460 mm. The number of viable cells correlated with the value of absorbance. The assay was performed in triplicate.

Protein analysis of by Western blot

Lysates from cells or tissues were used for Western blot analysis. Mammary epithelial cells were lysed in a 6-well dish in 0.5 ml of NP-40 IP buffer containing protease inhibitors and phosphatase inhibitors. SDS-PAGE was performed in Novex mini gel system with pre-cast 4-12% cells. Proteins were then transferred onto nitrocellulose membrane. After blocking with 5% non-fat milk for 1 hour, membranes were
incubated with primary antibodies overnight. Primary antibodies and their dilution used include: anti-Dab2 (1/2,000) (BD Transduction Laboratories, 610465), anti-Beta-actin (1/2,000) (BD Transduction Laboratories, 612657), anti-Sos1 (1/2,000) (BD Transduction Laboratories, 610096), anti-Grb2 (1/2,000) (BD Transduction Laboratories, 610112 and Santa cruz, sc-255), anti-E-cadherin (1/2,000) (BD Transduction Laboratories, 610405), anti-N-cadherin (1/2,000) (BD Transduction Laboratories, 610921), anti-Erk1/2 (1/2,000) (BD Transduction Laboratories, 610408), anti-phospho-Erk1/2 (1/2,000) (Cell Signaling, 4370), anti-cytokeratin 8 (1/2,000) (Developmental Studies Hybridoma Bank, Ames, IA), anti-Bcl-2 (1/2,000) (Cell Signaling, 3498), anti-Bcl-xl (1/2,000) (Cell Signaling, 2764), anti-cleaved caspase 3 (1/2,000) (Cell Signaling, 9664), anti-phospho-SMAD 2 (1/2,000) (Cell Signaling, 9510), anti-GC-globulin (1/2,000) (Abcam, ab65636), anti-F4/80 (1/2,000) (Abcam, 6640), anti-PCNA (1/2,000) (Santa Cruz, sc-56), and anti-Beta-casein (1/2,000) (Santa Cruz, sc-30041). (anti-estrogen and) The secondary antibodies were conjugated with horseradish peroxidase (HRP) and were used following the instruction from the manufacturer (Rabbit, mouse BioRad; Goat, Jaskson Immunolab). SuperSignal West Extended Duration Substrate (PIERCE) was used for chemoluminescence detection of protein bands.

**Co-immunoprecipitation**

Mammary epithelial cells in a 6-well dish were lysed with 0.5 ml of cold NP-40 IP buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) supplemented with protease inhibitors and phosphatase inhibitors. Cell lysates were centrifuged at 14,000 rpm for 20 min at 4oC to remove the nuclear fraction. The
supernatant was incubated with specific antibodies (10 μl of anti-Grb2 per 1 m of cell lysate) for 3 hours at 4°C. Immunoprecipitation was performed with Dynabeads protein G immunoprecipitation kit (Invitrogen). Protein G Dynabeads were added, and the mixtures were incubated for 1 hour. The beads were then collected by brief centrifugation and washed three times in IP buffer. Proteins bound to the beads were eluted in SDS-sample buffer and subjected to Western blot analysis.

**Immunohistochemistry and immunofluorescence**

Tissues were fixed with neutral buffered formalin, paraffin embedded and sectioned on a microtome at 5 μm thickness. Slides were deparaffinized in a graded ethanol series, washed in water and boiled in antigen retrieval solution (10 mM sodium citrate, pH 6.0) prior to staining. The sources are antibodies are: anti-adaptin-alpha anti-clathrin, anti-NPT2b, and anti-PMCA2. Other primary antibodies used for histology were the same as those used in immunoblotting. The dilution were: anti-Dab2 (1/2,000), anti-E-cadherin, anti-adaptin-alpha. Species-specific secondary antibodies were conjugated with the appropriate Alexa fluorochrome for simultaneous imaging of multiple antigens. DAPI (4’-6-diamidino-2-phenylindole) was used as a generic counterstain and applied in the terminal washing stages of the procedure. For immunohistochemistry, the secondary antibodies were HRP-conjugated (Vector Laboratories, CA, USA) and were detected by a DAB Peroxidase Substrate Kit (Vector Laboratories, CA, USA) followed by a hematoxylin counterstain.
Laser scanning confocal microscopy

All confocal imaging was performed with an inverted Zeiss LSM510/uv Axiovert 200M, laser scanning confocal microscope operated by Zeiss LSM software. Images were acquired with three sequential scan tracks. Objectives used included Plan-Apochromat (x63, 1.4 N/A) and Plan-Neofluar (x40, 1.3 N/A). Where necessary, images were contrast adjusted using Adobe Photoshop.

Ultrastructural evaluation by transmission electron microscopy

Tissues were fixed in a two-step procedure: initially by immersion in 2% glutaraldehyde, 100 mM sucrose, 0.5 M phosphate buffer, pH 7.3; followed by treatment with 2% osmium tetroxide in 1 M phosphate buffer. After a graded dehydration, the tissues were embedded by infiltration with Epon/Araldite resin (Electron Microscopy Sciences, Fort Washington, PA) and an overnight polymerization at 64°C. Thin sections were cut using a Leica Ultracut R ultramicrotome with a Diatome diamond knife, then mounted on copper grids and contrasted with 4% uranyl acetate and 0.25% lead citrate. The sections were examined in vacuo with electrons accelerated at 60 kV and focused using the magnetic lens of a Philips CM10 transmission electron microscope.

Propagation, genotyping, and husbandry of mouse models

We produced and used a dab2 flox mouse line constructed to delete both exons 3 and 4 of the dab2 gene, which leads to a null allele (Moore et al, 2013). From our previous study, the dab2 (f/f) mice were found to be indistinguishable from the wild type mice in the absence of Cre recombinase. To delete the dab2 gene conditionally, female
dab2 (f/f) mice were crossed with male dab2 (+/df):Sox2-cre or dab2 (+/df):Fabp4-cre to produce progenies for experiments. Litters with the dab2 (f/df):Sox2-cre genotype were designated as conditional knockouts (CKO), while dab2 heterozygous (HET) mice, dab2 (+/df):Sox2-cre were used as controls. Heterozygous dab2 mice from the same litters showed no detectable phenotypes and were used as controls for the conditional knockout mice. All mice comprised a genetic background predominately C57BL/6 although a minor 129/Sv contribution (originating from gene targeting in ES cells) persisted.

Sox2-cre mice (Tg(Sox2-cre)#Amc/J) (Hayashi et al., 2002) and Fabp4-cre mice (B6.Cg-Tg(Fabp4-cre)1Rev/J ) (He et al., 2003 ) were purchased from Jackson Laboratories. The mouse colonies were housed inside the barrier area of the mouse facility of University of Miami Miller School of Medicine and PCR genotyping was performed as described previously (Moore et al., 2013). Mice were routinely reared on a regular enriched diet, 5K20 chow pellets (10% fat, from LabDiets, Inc.). However, D12492 chow (Research Diets, Inc.) that contains 60% kcal from fat was used as a high fat diet (HFD) regimen for inducing weight gain/obesity, and D12450B (20% protein, 70% carbohydrate, and 10% fat) was used as normal control diet. Body weight was measured weekly using a lab balance. Animals were housed on a 12hr:12hr light:dark cycle with ad libitum access to food and water.

**Quantification of tissue composition**

The fat and lean tissues of Dab2 conditional knockout (CKO) and heterozygous (HET) littermates were determined non-invasively using the PIXImus small animal dual-X-
ray absorptiometry (DEXA) system (PIXImus™, GE Medical Systems, Fitchburg, WI: http://piximus.com/). Prior to analysis, the mice were anesthetized with an intraperitoneal injection of ketamine/xylazine cocktail, placed in the prone position such that the sub-cranial tissues could be scanned by the Lunar PIXImus Densitometer.

**Blood chemistry profile**

Mice were fasted for 4-6 hours and then anesthetized prior to tissue collection. Blood was collected by cardiac puncture, and the required tissues and fats were harvested. Following blood clot, serum samples were collected following spinning for 10 min at maximum speed in a bench top clinical centrifuge, and stored at -80°C until use. Lipid chemistry analysis was performed using a Vitros 250 chemistry analyzer (Johnson & Johnson) by the University of Miami Comparative Pathology Laboratory. The levels of serum cholesterol, triglyceride, and high-density lipoprotein (HDL) where determined colorimetrically. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were estimated as following: VLDL = 1/5 of triglycerides; LDL = total cholesterol minus HDL and VLDL.

**Preparation of primary cells and protein extracts**

Primary mouse embryonic fibroblasts (MEF) were isolated from E12.5 mouse embryos from crosses of between dab2 (f/f) females and dab2 (+/df):Sox2-cre males. Briefly, the head of dissected embryos was first removed and used for genotyping and the remainder was minced using a scalpel. After incubation for 10 min in 0.25% trypsin at 37oC, the mixture was pelleted by a brief centrifugation and plated in a T75 flask in DMEM media supplemented with 10% FBS. The cells from the outgrowth were
harvested and expanded. Western blot analysis of the cells was used to confirm the
genotype.

**Adipocyte differentiation assay and oil-O red staining**

Mutant and wild-type MEFs were used for adipocyte differentiation in culture. Monolayer cultures at about 50% confluency were first treated for 2 days with the “Induction Medium” (DMEM supplemented with 10% FCS, 8 µg/ml biotin, 4 µg/ml pantothenate, 0.5 mM 3-isobutyl-1-methylxanthine, 1µM dexamethasone, 10 µg/ml insulin, and 10 µM troglitazone). The induced cells were than cultured in “Differentiation Medium” (DMEM supplemented with 10% FCS, 8 µg/ml biotin, 4 µg/ml pantothenate, and 10 µg/ml insulin) for up to another 12 days to achieve differentiation to adipocytes. Mesenchymal stem cells (MSCs) were isolated from bone marrow and cultured as described (Soleimani and Nadri 2009). Briefly, cells were aspirated from bone marrow harvested from tibia and femur and cultured in DMEM with 15% FBS. After incubation overnight, non-adhesive cells were removed. After 2-3 passages, the remaining cells were MSCs and were used for differentiation through the standard protocol (Rosen, et al. 1999) of a 2-day induction and 7-day differentiation.

Embryonic stem (ES) cells of Dab2 positive and null were generated from mouse embryos as previously described (Moore, Cai et al. 2013). The ES cells were differentiated into adipocytes according to the standard procedure. Oil-O Red staining was performed to visualize the intracellular lipid droplets. Briefly, 0.5 g of Oil-O Red was dissolved in 100 ml isopropanol overnight with agitation. After sedimentation,
Oil-O Red solution and water was mixed in 6:4 ratios to making the working solution. The differentiated adipocytes were fixed in 4% PFA for 1 hour after washing 3 times with PBS, air dried, and incubation for 15 min with freshly filtered Oil-O Red working solution. After removing the Oil-O Red solution and washing 3 times by PBS, the cells were subject for microscopy imaging and quantification. The adipocyte associated Oil-O Red dye was extracted with 100% isopropanol and the absorbance was measure at the wavelength of 510 nm.

**Cytoplasmic and nuclear fractionation**

Proteins comprising the nuclear and cytoplasmic compartments were isolated from cultured cells using the NE-PER nuclear and cytoplasmic extraction reagents according to the protocol provided by the manufacturer (Thermo Scientific). Lamin A/C was used as a nuclear and Grb2 as a cytoplasmic markers in Western blot to validate the purity of the fractions.

**Histomorphometric quantification of adipocyte diameter**

The cell diameter of adipocytes was measured from histological slides stained with H&E using the MetaMorph Image Analysis Software. The red channel color of the original RGB images was selected and converted to a black and white image. The individual cell diameters was assayed were imported into a spread sheet format and further analyzed for average diameter.
**Co-immunoprecipitation and western blots**

Cells in a 6 well plate were lysed with 0.5 ml of cold NP-40 IP buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) with protease inhibitor cocktails and phosphatase inhibitors (Pierce Biotech). The nuclear fraction was removed by centrifuging at 14,000 rpm for 20 min at 4°C. The supernatant were collected and incubated with 10 μl of anti-Grb2 antibodies for 3 hours at 4°C, and immunoprecipitation was collected using Dynabeads protein G immunoprecipitation kit (Invitrogen). The beads were they collected by a magnet and washed three times with IP buffer. Antibodies and proteins bound to the bead were eluted by boiling in SDS-sample buffer and subjected to Western blot. Primary antibodies and their dilution used include: anti-Dab2 (1/5,000) (BD Transduction Laboratories, 610465), anti-Beta-actin (1/5,000) (BD Transduction Laboratories, 612657), anti-Sos1 (1/1,000) (BD Transduction Laboratories, 610096), anti-Grb2 (1/2,000) (BD Transduction Laboratories, 610112 and Santa Cruz, sc-255), anti-Erk1/2 (1/5,000) (BD Transduction Laboratories, 610408), anti-phosphoErk1/2 (1/1,000) (Cell Signaling), anti-PCNA (1/2,000) (Santa Cruz, sc-56), The secondary antibodies were conjugated with horseradish peroxidase (HRP) and were used (1/5,000 dilution) following the instructions from the manufacturer (rabbit and mouse secondary antibodies from BioRad; goat secondary antibodies from Jackson ImmunoResearch). SuperSignal West Extended Duration Substrate (Pierce Biotech) was used for chemoluminescence detection of proteins.
**Histology, immunofluorescence and confocal microscopy**

Tissues were fixed with formalin, embedded in paraffin, and sectioned with a microtome at 6 μm thickness. Slides were deparaffinized in a graded ethanol series, washed in water and boiled in antigen retrieval solution (10 mM sodium citrate, pH 6.0) prior to addition of antibodies. Primary antibodies are: anti-PPARγ, anti-C/EBPα, anti-Dab2, and anti-phospho-Erk1/2. Species-specific secondary antibodies were conjugated with the appropriate Alexa fluorochrome for simultaneous imaging of multiple antigens. DAPI (4’-6-diamidino-2-phenylindole) was used as a generic counterstain and applied in the terminal washing stages of the procedure. For lipid droplet staining, BODIPY 495/503 (Invitrogen) was diluted in 1:1,000 and added at the same time as secondary antibodies. In detection of multiple antigens with fluorescence microscopy, Bodipy was imaged last to avoid the persistent fluorescence upon stimulation of the dye. Confocal imaging was performed with an inverted Zeiss LSM510/uv Axiovert 200M, laser scanning confocal microscope operated by Zeiss LSM software. Images were acquired with three sequential scan tracks. Objectives used included Plan-Apochromat (x63, 1.4 N/A) and Plan-Neofluar (x40, 1.3 N/A). For immunohistochemistry, the secondary antibodies were HRP-conjugated (Vector Laboratories, CA, USA) and were detected by a DAB Peroxidase Substrate Kit (Vector Laboratories, CA, USA) followed by a hematoxylin counterstain.

**Quantitative RT-PCR for the analysis of gene expression**
Total RNA was harvested from MEF cells using the Qiagen RNAeasy kits and cDNAs were prepared by reverse transcription with iScript™ cDNA Synthesis Kit all according to the manufacturer’s instructions. Quantitative RT-PCR was performed using CFX Connect™ Real-Time PCR Detection System with 2X SYBR® Green Supermix and primer pairs. The primers used were: GAPDH: (sense) AGT GGA GAT TGT TGC CAT CAA CGA CC; and (antisense) GGA CTG TGG TCA TGA GCC CTT CC; PPARγ1: (sense) TGA AAG AAG CGG TGA ACC ACT G, and (antisense) TGG CAT CTC TGT GTC CAA CCA TG; PPARγ2: (sense) GTT TTA TGC TGT TAT GGG TG, and (antisense) GTA ATT TCT TGT GAA GTG CTC ATA G; C/EBPα: (sense) GCC ATG GCC TTG ACC AAG GAG, and (antisense) GAA CAG CAA CGA GTA CCG GGT A; leptin: (sense) GGG ATG GCT CTT ATC TCT ACT TGC T, and (antisense) CAC CAG GCT CCC AAG AAT CAT GTA.
Reference


