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## ***c-fos* elimination compensates for *disabled-2* requirement in mouse extraembryonic endoderm development**

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### **Abstract**

Disabled-2 (Dab2) is expressed in primitive endoderm cells as they are differentiating from the inner cell mass and *dab2* deficiency in mice results in lethality at E5.5-E6.5 due to the disorganization of the endoderm layers. Here we show that Dab2 suppresses c-Fos expression in endoderm cells. A morphological normal primitive endoderm layer was observed in putative E5.5 *dab2* (–/–):*c-fos* (–/–) embryos, indicating that the primitive endoderm defect due to the loss of Dab2 is rescued by deletion of *c-fos* gene. The lethality of the double knockout embryos delayed until E9.5-E10.5 and the defective embryos failed to undergo organogenesis. We conclude that Dab2 plays a role in epithelial organization by suppression of c-Fos expression and suggest that unsuppressed c-Fos can lead to disruption of primitive endoderm epithelial organization, yet an additional *dab2* function is required for later organogenesis.

### **Keywords**

Disabled-2 (Dab2); c-Fos; primitive endoderm; cell surface positioning; cell positioning/organization; epithelial polarity; MAPK; mouse embryos

## **INTRODUCTION**

Disabled-2 (Dab2) is one of the two mammalian orthologs of the *drosophila* Disabled (Gertler *et al.*, 1989; Xu *et al.*, 1995; Howell *et al.*, 1997). Mouse *dab1* is expressed mainly in the brain and studies of mutant and knockout mice suggest that Dab1 functions in brain cell positioning organization (Howell *et al.*, 1997a,b; Sheldon *et al.*, 1997). Dab2 was isolated as a signaling phosphoprotein and found to act as a putative tumor suppressor (Xu *et al.*, 1995; Mok *et al.*, 1998; Fazili *et al.*, 1999). The cellular function of Dab2 is established to be an adaptor protein in endocytosis (Mishra *et al.*, 2002; Bonifacino *et al.*, 2003). Dab2 associates with endocytic cargos by binding to adaptin and clathrin (Morris and Cooper 2001; Mishra *et al.*, 2002), and also to cargo passengers including trans-membrane glycoproteins such as low-density lipoprotein receptor family members (Trommsdorff *et al.*, 1999; Oleinikov *et al.*, 2000; Morris and Cooper 2001) and integrins (Wang *et al.*, 2001; Huang *et al.*, 2004). The C-terminus of Dab2 protein binds to the actin-based, minus end-

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directed motor protein myosin VI (Inoue *et al.*, 2002; Morris *et al.*, 2002a; Hasson, 2003); thus Dab2 mediates the attachment of cargos to motor proteins.

Dab2 is expressed in many epithelial cell types and was suggested to have a role in epithelial organization as a mode of action in tumor suppression (Fazili *et al.*, 1999; Sheng *et al.*, 2000; Yang *et al.*, 2002a), paralleling the role of *dab1* in neuronal cell positioning and organization (Howell *et al.*, 1997b; Sheldon *et al.*, 1997). The role of *dab2* gene in epithelial organization is supported by the primitive endoderm disorganization in the early embryonic lethal phenotype of *dab2* ( $-/-$ ) mouse embryos (Yang *et al.*, 2002b, 2007). In E5.5 *dab2* homozygous deficient embryos, cells of the visceral endoderm, the epithelial cell type of the early embryos, mix within the interior rather than align as a layer covering the inner cell mass (Yang *et al.*, 2002b, 2007). The role of Dab2 in mediating directional transporting of endocytis cargos to establish apical polarity is suggested to be one mechanism for surface positioning of the endoderm cells (Yang *et al.*, 2007).

In conditional targeted knockout mice using Meox2-cre (Tallquist and Soriano, 2000; Morris *et al.*, 2002b), the *dab2* gene was not deleted in the extraembryonic endoderm, but was deleted in a mosaic fashion in cells of the embryo proper. These *dab2* mosaic mutant mice completed development. Defects in kidney function, however, were noted (Morris *et al.*, 2002b). Dab2 is expressed in several additional tissues (Fazili *et al.*, 1999), but the potential developmental and biological roles of Dab2 in these tissues are not yet well studied.

In cultured cells, transfection/ expression of Dab2 resulted in suppression of c-Fos expression, uncoupling it from MAPK activation (He *et al.*, 2001; Smith *et al.*, 2001a). Dab2 may do so by limiting the entry of the activated MAPK into the nucleus (Smith *et al.*, 2001a, 2004), where c-Fos transcriptional activation is a key target of the kinase. The role of Dab2 in regulating c-Fos expression has been shown in the retinoic acid-induced endoderm differentiation of embryonic carcinoma and stem cells (Smith *et al.*, 2001a,b, 2004). *c-fos*, an immediate early gene, is a component of the AP-1 transcription complex (Curran and Franza, 1988). *c-fos* is dispensable for cell growth and development in mice, although *c-fos* deficient mice develop severe osteopetrosis such as foreshortening of the long bones, ossification of the marrow space, and absence of tooth eruption shortly after birth (Johnson *et al.*, 1992; Hu *et al.*, 1994). *c-fos* is required for malignant tumor growth (Saez *et al.*, 1995), and overexpression of c-Fos can disrupt mammary epithelial organization (Reichmann *et al.*, 1992). In a genetic analysis of anchor cell invasion of vulval epithelium in *C. elegans*, a key role for Fos in the disruption of epithelial basement membrane and invasion was demonstrated (Montell, 2005; Sherwood *et al.*, 2005). The genetic approach also identified three Fos targets: a matrix metalloproteinase; an extracellular matrix component (hemimentin); and a protocadherin. These proteins were shown also to be critical for epithelial invasion and disruption of basement membrane in the formation of vulval structure (Montell, 2005; Sherwood *et al.*, 2005).

In the current study, we sought to verify the *in vivo* relevance of the observation in cell culture that Dab2 suppresses c-Fos expression. Thus, by crossing *dab2* and *c-fos* knockout mice, we investigated the influence of *c-fos* deletion on the visceral endoderm organization of Dab2 null embryos. The result confirmed the importance and *in vivo* activity of Dab2 in regulating c-Fos expression in the organization of visceral endoderm of early mouse embryos.

## RESULTS

### Dab2 Down Regulation Enhances c-Fos Expression in Differentiated Embryonic Stem Cells

First, we examined whether Dab2 is essential for suppression of c-Fos expression in cultured mouse embryonic stem (ES) cells. Proliferating ES cells are negative for Dab2 and express c-Fos (Fig. 1A). Following treatment with retinoic acid for 4 days, the ES cells differentiated into primitive endoderm-like cells (Smith *et al.*, 2004). In the differentiated cells, Dab2 expression is induced, and c-Fos expression is suppressed (Fig. 1A), consistent with previous studies in ES and F9 embryonic carcinoma cells (Smith *et al.*, 2001a,b,2004). When Dab2 expression is suppressed by siRNA in the differentiated cells, c-Fos expression is recovered (Fig. 1B,C), indicating Dab2 is essential for suppression of c-Fos expression in ES-derived primitive endoderm cells. In empty vector-transfected cells, retinoic acid induced endoderm differentiation and Dab2 expression is positive in the majority of cells (Fig. 1B), and only less than 15% of cells were undifferentiated (Dab2-negative) and expressing c-Fos (Fig. 1B, left panel). In parallel experiments, about 60% of the siRNA-Dab2 suppressing vector-transfected cells were Dab2-negative and showed high c-Fos expression (Fig. 1B, right panel). We consider that Dab2 was suppressed by siRNA in most of these cells, since the transfection efficiency was estimated to be 50% to 60%. The Dab2-siRNA vector was shown to be highly efficient in suppressing Dab2 expression in a previous report (Yang *et al.*, 2007).

The close correlation between c-Fos expression and Dab2 suppression is shown by another example (Fig. 1C), of differentiated culture containing both Dab2-positive/c-Fos-negative (arrowhead), and Dab2-negative but c-Fos-positive (arrow) cells. Thus, c-Fos expression is suppressed in ES cells upon differentiation as report previously (Smith *et al.*, 2001, 2004), and Dab2 is required for c-Fos suppression in these differentiated ES cells.

A simple statistical analysis was performed on the immunofluorescence images of the fractions cells with positive or negative Dab2 and c-Fos staining to compare vector and siRNA-Dab2-plasmid transfected cells (Table 1). The fraction of cells of either positive or negative for both Dab2 and c-Fos staining is in the range of 6% to 18%, and it is not statistically significant for difference between vector control or Dab2-suppressed cells. However, the shift from Dab2-positive/c-Fos negative in control (around 70%) to c-Fos-positive/Dab2-negative (around 60%) in Dab2-suppressed cells, is statistically significant (Table 1). Thus, we conclude that Dab2 expression has a negative impact on c-Fos expression in ES cells, consistent with previous reports (He *et al.*, 2001;Smith *et al.*, 2001a).

### c-fos Gene Deletion Rescues Endoderm Development in Dab2 Null Embryos

To examine the in vivo and biological relevance of the regulation of c-Fos expression by Dab2 that observed in cultured cells, we tested the genetic relationship of *dab2* and *c-fos* genes in mice. Specifically, we determined if elimination of *c-fos* gene could rescue the embryonic lethal phenotype of *dab2* knockout (Yang *et al.*, 2002b, 2007) by crossing *c-fos* and *dab2* heterozygous mice and examining the presence of offspring with *dab2* (-/-):*c-fos* (-/-) genotype. In 181 progenies obtained from intercrosses between *dab2* (+/-):*c-fos* (+/-) littermates examined, we found no newborn pups with the *dab2* (-/-) genotype (Table 2). It is predicted that 1 in 16, or about 11 of the offspring among the total of 181 should be such genotype if the mutant mice could complete development. In this relatively small number of progenies analyzed, an under-representation of *c-fos* (-/-) pups was observed either in the *dab2* wildtype (0.62/16 instead of 1/16 expected) or heterozygous (1.1/16 instead of 2/16 expected) background, consistent with the reported reduction of embryonic viability of the

*c-fos* knockout (Johnson *et al.*, 1992). Instead, proportion of the progenies with *c-fos* (+/-) genotypes was increased.

Thus, deletion of *c-fos* is not sufficient to rescue the complete development of *dab2* (-/-) mutants. Nevertheless, when examine prenatal embryos (Table 3), we identified the presence of *dab2* (-/-):*c-fos* (-/-) embryos that showed abnormal morphology at E8.5 to E10.5 stages in a ratio approximating the Mendelian prediction (1/16 is predicted to be double knockouts) (Table 3). However, no *dab2* (-/-) embryos of *c-fos* (+/-) or (-/-) embryos were found in these developmental stages. *dab2* (-/-) embryos normally do not exist beyond E6.5 (Yang *et al.*, 2002, 2007). Thus, it appears that the *c-fos* knockout background delays the embryonic lethality phenotype of *dab2* (-/-) mice.

The E5.5 embryos from crosses between *dab2* (+/-):*c-fos* (+/-) mice were analyzed by immunostaining for Dab2 to identify genotypes as *dab2* (-/-) or *dab2* (+/+) and *dab2* (+/-). The Dab2-positive embryos exhibit the GATA4-positive visceral (arrow) and parietal (arrowhead) cells (Fig. 2A). Most Dab2-negative embryos showed the disorganization of GATA-4-positive endoderm cells (Fig. 2B), as reported previously for *dab2* knockout phenotype (Yang *et al.*, 2002, 2007). Several embryos were identified as *dab2*-negative but maintained organization and surface positioning of GATA4-positive endoderm cells (Fig. 2C). Such embryos were observed only from *dab2* (+/-):*c-fos* (+/-) parents but were never detected in embryos from *dab2* (+/-):*c-fos* (+/+) parents. We interpret that these embryos are *dab2* (-/-):*c-fos* (-/-) genotype (Though such small embryos were not suitable for genotyping of *c-fos* status directly by PCR. Also the available anti-c-Fos antibody is not suitable for staining of fixed and paraffin-embedded tissues). The existence of *dab2* (-/-):*c-fos* (-/-) embryos in later developing stages was confirmed by direct genotyping using PCR of *dab2* and *c-fos* mutations (Table 3). The presence of later double mutant embryos supports the interpretation that of Dab2-negative but morphological normal E5.5 embryos have *dab2* (-/-):*c-fos* (-/-) genotype. Thus, we conclude that deletion of *c-fos* rescues the endoderm developmental defect of *dab2* (-/-) embryos and postpones the lethality to a later stage.

### Dynamic Dab2 Expression Pattern in Early Embryonic Development

During mouse development, Dab2 is first expressed at E4.5 in the primitive endoderm cells and is then expressed only in extraembryonic tissues but not embryo proper until E8.5 to E9.0 stages (Yang *et al.*, 2002). The *dab2* mutation was made by an in-frame insertion of lacZ, allowing us to follow beta-galactosidase activity as a reporter for Dab2 expression in *dab2* (+/-) mice (Yang *et al.*, 2002). Essentially no beta-galactosidase staining was observed in embryos (only in extraembryonic yolk sac) prior to E9.0 (Fig. 3A). At a stage around E9.0, some of the *dab2* (+/-) embryos express beta-galactosidase only in the yolk sac endoderm (Fig. 3A, left), while some littermates express beta-galactosidase in discrete sites within the embryo proper (for example, the septum transversum) (Fig. 3A, right) in addition to the yolk sac.

Starting from E9.5, beta-galactosidase staining becomes more widely distributed, including the strong staining of the cardiac tissue in E9.5 and E10.5 stages (Fig. 3A). At E11.5 and E13.5 (Fig. 3A), beta-galactosidase activity is present in most parts of the embryos. Sectioning of the embryos provides more precise localization of the staining (Fig. 3B). As shown in an E9.0 embryo, only extraembryonic yolk sac shows strong Dab2 immunostaining (Fig. 3B, indicated by a red "\*" in the left panel and shown in a higher magnification in the middle panel). The beta-galactosidase staining of E9.0 yolk sac (Fig. 3A) is shown in a higher magnification of a section for comparison with Dab2 immunostaining (Fig. 3B, right panel). In mice, Dab2 mRNA expression is high in kidney (Fazili *et al.*, 1999). The kidney tubule epithelia are strongly positive for Dab2 or beta-

galactosidase activity (Fig. 3C), indicating the pattern of beta-galactosidase staining in the *dab2* (+/-) mice is consistent with the presence of Dab2 protein.

Thus, these observations suggest that Dab2 is first expressed in the embryo proper at around E9.0, initially in the hepatic primordial region, next in the cardiac area, and then becomes widely distributed throughout the embryos.

### **c-fos Elimination Delays Embryonic Lethality of Dab2 Null Embryos**

Overcoming extraembryonic endoderm disorganization and escaping lethality at E5.5-E6.5 stages, the *dab2* (-/-):*c-fos* (-/-) embryos persisted to E9.5 to E10.5 stages (Table 3). Nevertheless, the double mutant embryos were found to have greatly disturbed morphology. Over the period of about 2 years when we continually produced and analyzed embryos from mating between *dab2* (+/-):*c-fos* (+/-) mice, a large range of variable morphology and phenotypes of the double mutant embryos at E9.5 to E10.5 stages were observed. In the example of an E9.5 mutant embryo shown in Figure 4A and B, the abnormal embryo identified as *dab2* (-/-):*c-fos* (-/-) by PCR (Fig. 4C) was much smaller than a *dab2* (+/+) littermate. The endoderm area of the abnormal embryo stained positive for beta-galactosidase/Dab2 (Fig. 4B). In another striking example of an E10.5 *dab2* (-/-):*c-fos* (-/-) embryo (Fig. 5), the abnormal embryo appeared unusually trim and lacked an apparent heart or other organs, which was confirmed by sectioning through the entire embryo (Fig. 5B). The *dab2* (-/-):*c-fos* (-/-) genotype of this embryo was identified by PCR (Fig. 5C). This phenotype resembles a reported acardia embryo when both GATA4 and GATA6 are deleted (Zhao *et al*, 2008). Thus, deletion of *c-fos* delays the embryonic lethality of *dab2* (-/-) genotype, which in turn reveals a role of *dab2* in organogenesis when Dab2 is started to be expressed in hepatic primordial region at around E9.0.

This analysis indicates that Dab2 has a critical role in suppressing c-Fos expression in vivo, and loss of Dab2-mediated regulation of c-Fos expression may be a cause of extraembryonic disorganization in Dab2-null embryos. Another activity of Dab2 in addition to c-Fos suppression is required for organogenesis at a later stage (E9.0) when Dab2 expression is initiated in the embryo proper, coincident with the morphogenesis of heart and liver.

## **DISCUSSION**

Previously, Dab2 was found to uncouple MAPK activation and c-Fos expression in differentiated ES and embryonic carcinoma cells in cultures (Smith *et al*, 2001a). In this study we examined the genetic relationship between *dab2* and *c-fos* in early mouse development and the result suggests that the regulatory relationship between Dab2 and c-Fos exists in vivo. We found that absence of *c-fos* can partially rescue the early embryonic lethality of Dab2 null embryos. Thus, suppression of c-Fos expression by Dab2 is required for the formation and maintenance of extraembryonic endoderm organization in early embryos. However, *dab2* (-/-):*c-fos* (-/-) embryos still die around E9.5 to E10.5, suggesting that Dab2 has additional, c-Fos-independent requirement in organ development in embryogenesis.

### **Role of Dab2 and c-Fos in Cell Mobility and Epithelial Organization**

In cell culture studies, *c-fos* expression was suppressed by Dab2 expression (He *et al*, 2001; Smith *et al*, 2001a). The current study confirms that elimination of Dab2 can dis-regulate c-Fos expression in differentiated ES cells in culture. Additionally, elimination of c-Fos can rescue endoderm disorganization of Dab2-null embryos, suggesting that c-Fos contributes to the disorganization of endoderm in Dab2-null embryos. This conclusion on the biological function of c-Fos is consistent with earlier studies that c-Fos over-expression contributes to

disorganization of mammary epithelium (Reichmann *et al*, 1992) and that c-Fos contributes to tumor malignancy (Saez *et al*, 1995).

Dab2 has a role in the establishment and maintenance of epithelial polarity through its cellular function in directional cargo endocytic trafficking. Previously, the role of Dab2 in establishing and maintaining cell polarity is also suggested to be critical in endoderm epithelial positioning and organization (Yang *et al*, 2007). Likely, Dab2 may function in endoderm epithelial organization through by two avenues: the establishment and maintenance of apical polarity to position the cells and the suppression of c-Fos to reduce mobility of the endoderm epithelial cells.

### Mechanism for Regulation of c-Fos Expression by Dab2

The current study focus on verifying the *in vivo* relevance and the genetic relationship of Dab2 and c-Fos expression and has not provided direct insight into the mechanism of how Dab2 may regulate c-Fos. One likely hypothesis is that Dab2 controls c-Fos expression by regulating the nuclear entry of the activated MAPK. Although in cell culture studies, MAPK seems to readily enter nucleus upon activation, nuclear entry of the activated MAPK is regulated *in vivo* (Kumar *et al*, 2003). In *Drosophila*, activated MAPK is restricted to cytoplasm, termed as “cytoplasmic hold”, until certain event triggers the entry of the activated MAPK into the nucleus to induce cell fate determination (Kumar *et al*, 2003; Marenda *et al*, 2006). The “cytoplasmic hold” of MAPK in *Drosophila* was accounted for by the localization of importin 7/Moleskin to the cytoplasmic instead of nuclear (Vrailas *et al*, 2006). Importin 7/Moleskin is required for MAPK nuclear entry, and its relocalization to the nuclear pores concurs with nuclear entry of MAPK (Vrailas *et al*, 2006).

Thus, based on the understanding of nuclear entry of MAPK in *Drosophila*, a possible model in mammalian cells is that Dab2 may restrict MAPK nuclear entry by mediating the trafficking of cargos containing importin 7 (or other importin member in mammalian cells required for MAPK import) away from the nuclear pores, and thus, preventing the efficient entry of the activated MAPK into nucleus to activate the expression of c-Fos. These possibilities await experimental examination.

In sum, the current study demonstrates the *in vivo* and biological relevance of Dab2 in regulating c-Fos expression and function, and suggests presence of additional c-Fos-independent role of Dab2 in organogenesis in embryonic development.

## EXPERIMENTAL PROCEDURES

### *dab2*-, and *c-fos*-deficient Mice and Embryo Analysis

Two lines of *dab2* knockout mice were established previously (Yang *et al*, 2002b) and the colonies were maintained by inbreeding in the Animal Facility of Fox Chase Cancer Center. The two lines have given identical phenotypes and will not be distinguished here. For embryo analysis, timed-matings were set up between 10 pairs of *dab2* ( $-/-$ ) mice of around 3 months old. The next day, vaginal plugs were examined as an indication of pregnancy. At scheduled days after conception, the pregnant females were sacrificed to harvest uteri for fixing and paraffin embedding. At earlier stages (earlier than E6.5), *dab2* genotyping was performed by morphological examination and/or Dab2 immunostaining of the embryos. Dab2-positive littermates in the same uterus were used as positive controls.

The founder pair of *c-fos* mice (B6.129 $\times$ 1-*Fos*<sup>tm1Pa/J</sup>) (Johnson *et al*, 1992) was purchased from the Jackson Laboratory and the mouse colony was maintained in C57/B6 background. The genotyping protocol by PCR amplification of tail genomic DNA of from the Jackson Lab

([http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol\\_id=1699](http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=1699)) was followed.

Mating pairs were used to establish a *dab2* (+/-):*c-fos* (+/-) colony and were studied over a 2-year period. Embryos from timed mating of *dab2* (+/-):*c-fos* (+/-) parents were harvested for analysis.

### Histology and Immunohistochemistry

Uteri with embryos at different developmental stages were collected. The tissue samples were formalin-fixed and paraffin-embedded. Sections (5  $\mu$ m) were cut and adhered to positively charged slides. Routine H&E staining was applied. Immunohistochemistry using a primary antibody to Dab2 (BD Biosciences) diluted 1:400 was performed as previously described (Yang *et al.*, 2002b, 2007). For GATA4 staining, the sections were subjected to antigen retrieval by steaming for 20 min in citrate buffer (10 mM). Rabbit polyclonal anti-GATA4 antibodies (Santa Cruz Biotechnology, Inc.) were used (1:800 dilution).

### Immunofluorescence Microscopy

Cells were plated on 22  $\times$  40 mm cover slides in 6 well dishes and fixed with 4% paraformaldehyde when they had reached 60% confluence. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS and blocked with 3% BSA in PBS containing 0.1% Tween-20 (room temperature for 30 min). Dab2 antibodies were used at 1:200 dilution in 1% BSA in PBS containing 0.1% Tween-20 and incubated for 2 hours. c-Fos polyclonal antibodies (Santa Cruz Biotechnology, Inc.) were used at a 1:200 dilution also. The cellular localization of the antigens was revealed by fluorescein or Texas Red conjugated secondary antibodies (Jackson Immuno Research lab, West Grove, PA) at 1:200 dilution. The secondary antibodies were: donkey anti-mouse IgG conjugated with Texas Red and donkey anti-rabbit IgG conjugated with Fluorescein. Nuclei were marked by DAPI staining. The Nikon Eclipse E 800 epifluorescence microscope with 60  $\times$  oil immersion objective linked to a Roper Quantix CCD (charged coupled device) camera were used for observation and image acquisition. A Nikon Eclipse E800 fluorescence microscope with 60  $\times$  water immersion objective linked to a Bio-Rad Radiance 2000 LSCM (laser scanning confocal microscope) camera was also used to examine the slides. Images were overlaid using Adobe Photoshop.

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### The abbreviations used are

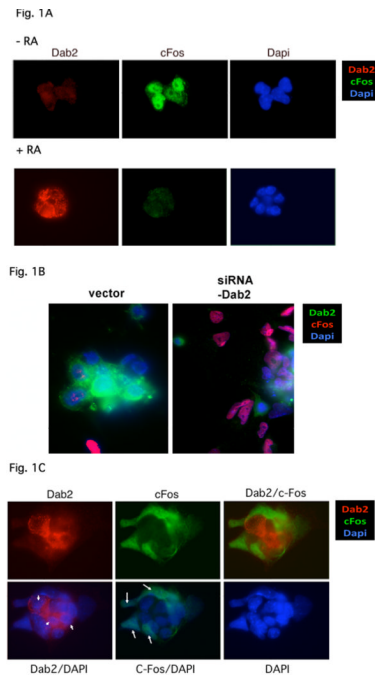
<b>Dab2</b>	Disabled-2, protein
<b>dab2</b>	Disabled-2 gene
<b>ES cells</b>	embryonic stem cells
<b>RA</b>	retinoic acid

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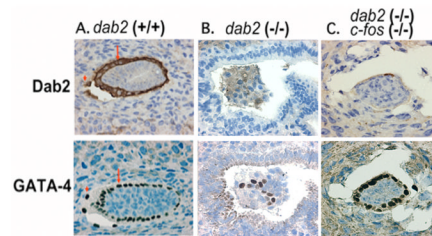


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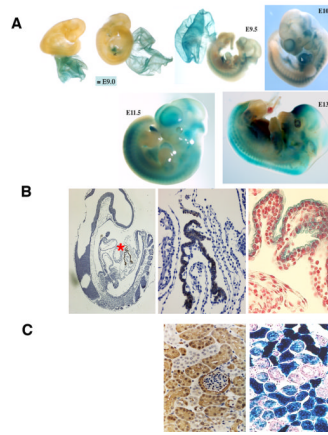
**Fig. 1.**

Dab2 is required for suppression of c-Fos expression in endoderm cells derived from differentiation of ES cells. **(A)** ES cells were treated with or without 1  $\mu$ M retinoic acid (RA) for 3 days to induce endoderm differentiation. The cells were then cultured without serum for 18 hours  $\pm$  RA, stimulated with 15% serum for 90 min, and fixed for immunostaining for Dab2 (red), c-Fos (green), or DAPI staining (blue). **(B)** On day 1, ES cells were transfected with siRNA-Dab2 vector and simultaneously treated with or without RA for 3 more days. The cells were cultured in medium containing low serum (1%) overnight and were then stimulated with 10% serum for 90 min and fixed for immunostaining for Dab2 (green), c-Fos (red), or for DAPI (blue). The cell numbers with various combinations of Dab2 and C-Fos expression were counted based on these images, and were used for statistical analysis as summarized in Table 1. **(C)** Dab2 (red) and c-Fos (green) expression in RA-treated ES cells transfected with the Dab2-siRNA suppressing vector. The cells are a mixture of with (arrow) or without (arrowhead) transfection/Dab2 suppression.



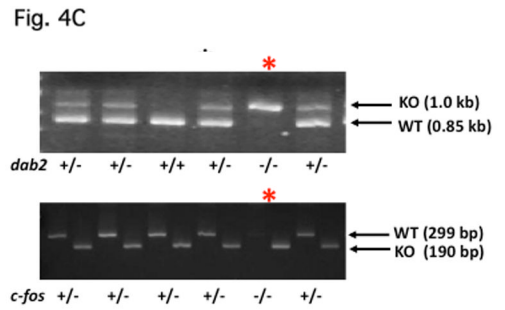
**Fig. 2.**

*c-fos* gene deletion rescues the endoderm disorganization in E5.5 *dab2* ( $-/-$ ) embryos. E5.5 embryos of various genotypes were stained with Dab2 or GATA-4 to determine protein expression (for *dab2* genotyping) and organization of the visceral endoderm cells. **(A)** An example of a wildtype embryo: the visceral endoderm cells are positive for GATA-4 and Dab2, and are organized into an epithelium covering the embryos. **(B)** An example of *dab2* ( $-/-$ ) embryo from a timed-mating between *dab2* ( $+/-$ ) mice. Lack of Dab2 immunostaining indicates it is a *dab2* ( $-/-$ ) embryo. Note that the GATA-4-positive endoderm cells are disorganized and fail to form a surface endoderm layer. **(C)** An example of a presumed *dab2* ( $-/-$ ):*c-fos* ( $-/-$ ) embryo. Dab2 staining is negative indicating a *dab2* ( $-/-$ ) genotype, while GATA-4-positive visceral endoderm cells appear to organize into an epithelium.



**Fig. 3.**

Dab2 expression in mouse embryos traced by lacZ reporter. *dab2* (+/-) mouse embryos and tissues, in which an in-frame fusion of lacZ cDNA after the first 27 amino acids of the *dab2* gene replaced the rest of the Dab2 protein, were used to trace Dab2 expression by staining for beta-galactosidase activity. (A) Representative embryos following beta-galactosidase activity staining are shown. Two representative E9.0 *dab2* (+/-) embryos from the same litter are shown, that the extraembryonic tissues were stained. One embryo shows no staining in the definitive embryonic tissue and the other embryo shows a unique site of staining. Additional examples of beta-galactosidase staining in *dab2* (+/-) E9.0, E9.5, E10.5, E11.5, and E13.5 embryos are shown. (B) Immunostaining of Dab2 in a section of an E9.0 embryo shows positivity only in extraembryonic yolk sac (left panel). The area indicated by a red "\*" is shown in a higher magnification (middle panel). A section shows the positive beta-galactosidase staining of the extraembryonic yolk sac from an E9.0 embryo (right panel). The section was counterstained with fast red. (C) The patterns of Dab2 immunostaining and beta-galactosidase activity staining of a kidney from 3-week old *dab2* (+/-) mouse are shown for comparison.



**Fig. 4.** Examples of E9.5 *dab2* (-/-) : *c-fos* (-/-) embryos. Embryos from timed-matings between *dab2* (+/-) : *c-fos* (+/-) mice were analyzed. Following photographing under a microscope, part of the embryonic tissues (often the extraembryonic tissues) was used for genotyping by PCR. (A) Comparison of E9.5 embryos enclosed in extraembryonic tissue from a timed-mating of mice. A *dab2* (+/+):*c-fos* (-/-) and a *dab2* (-/-):*c-fos* (+/-) embryos are shown. (B) The embryos were dissected from the extraembryonic tissue and stained with beta-galactosidase. (C) Genotyping by PCR to identify the morphologically abnormal embryo, indicated by “\*”, as *dab2* (-/-) : *c-fos* (-/-).

Fig. 5A

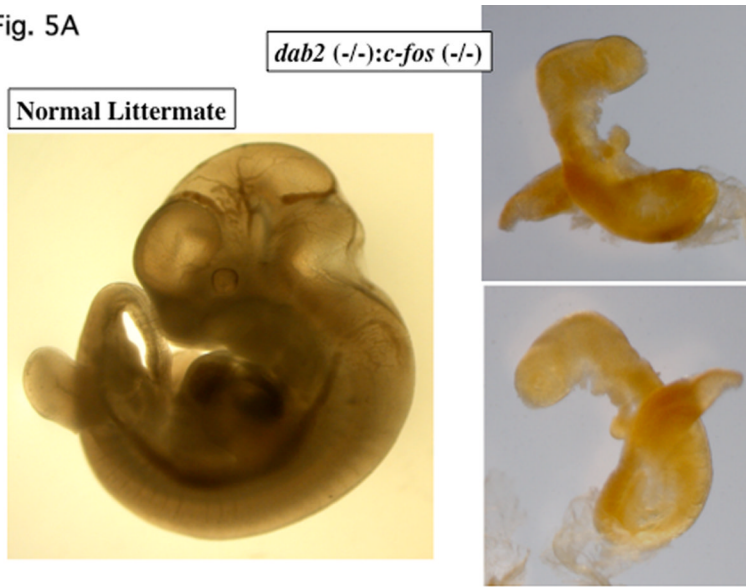


Fig. 5C

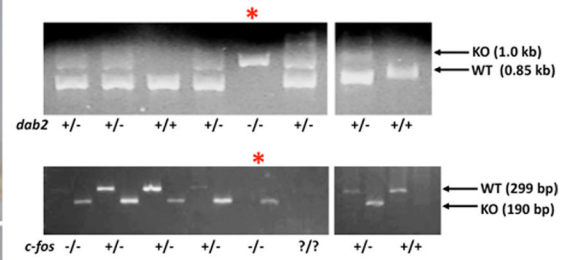


Fig. 5B

E10.5 Normal littermate

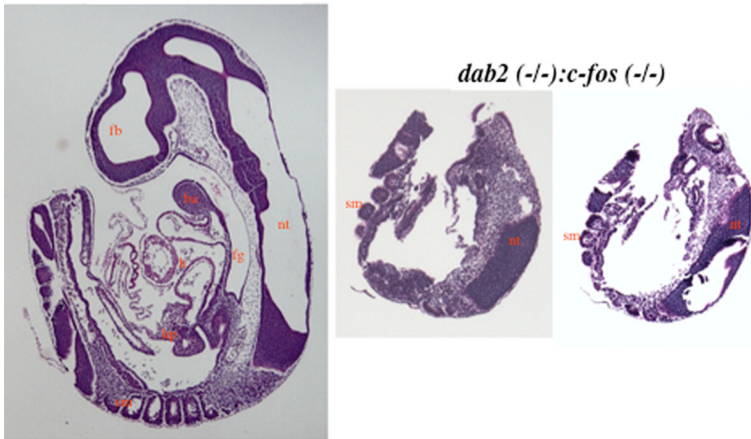


Fig. 5.

Delay of lethality to E10.5 of *dab2* (*-/-*) embryos in the *c-fos* null background. Embryos from timed-mating between *dab2* (*+/-*):*c-fos* (*+/-*) mice were analyzed. Following photographing under a microscope, part of the embryonic tissues (often the extraembryonic tissues, the yolk sac) was used for genotyping by PCR. (A) A pair of normal and abnormal (identified as *dab2* (*-/-*):*c-fos* (*-/-*)) embryos is shown. The abnormal embryo was photographed on both sides to show a lack of apparent organs. (B) The abnormal E10.5 embryo was sectioned and stained with H&E to compare with a wildtype littermate control. The abnormal embryo was sectioned through and two representative sections around the midpoint are shown. (C) A small fragment of each embryo was used in PCR amplification of *dab2* (wildtype, 1.0 kb; knockout, 0.85 kb) and *c-fos* (wildtype, 299 base pair; knockout, 190 base pair) genes for genotyping. The morphologically abnormal embryo, indicated by “\*”, was identified as *dab2* (*-/-*): *c-fos* (*-/-*) genotype. **Abbreviations in Figures:** ba, branchial arch; ca, cardiac mesoderm; en, endoderm; fg, foregut diverticulum; h, heart; hp, hepatic primordial; nt, neural tube; ov, optic vesicle; sm, somites; st, septum transversum.

**Table 1**

Fluorescence image analysis of the relationship between c-Fos and Dab2

Expression <i>dab2</i>	<i>c-fos</i>	vector-transfected			siDab2-transfected			p-value
		#1	#2	#3	#1	#2	#3	
+	+	2 (12%)	0 (0%)	2 (6%)	4 (18%)	2 (6%)	3 (10%)	0.34
-	+	1 (6%)	0 (0%)	3 (9%)	12 (54%)	20 (62%)	22 (73%)	0.031
+	-	12 (70%)	6 (86%)	23 (72%)	4 (18%)	5 (16%)	2 (7%)	0.005
-	-	2 (12%)	1 (14%)	4 (12%)	2 (9%)	5 (16%)	3 (10%)	0.68
<b>Total cell#</b>		17	7	32	22	32	30	

The retinoic acid-differentiated ES cells were transfected with empty vector or siRNA Dab2-suppressing vector and stained for Dab2 and c-Fos. Three fields each of vector- or siDab2 suppression plasmid-transfected cells were counted for c-Fos+ Dab2+, c-Fos+ Dab2-, c-Fos- Dab2+, and c-Fos- Dab2- cells. The staining was called for either positive or negative, ignoring relative intensity. The percentage of cells in each category was calculated and used for statistical analysis. The p-value for the difference between each group was calculated by student T-test ([http://www.physics.csbsju.edu/stats/t-test\\_bulk\\_form.html](http://www.physics.csbsju.edu/stats/t-test_bulk_form.html)). *P* < 0.05 is considered statistically significant.

**Table 2**PCR genotyping of progenies from intercrosses between *dab2* (+/-): *c-fos* (+/-) littermates

<i>Genotypes dab2 : c-fos</i>		<b>expected ratio</b>	<b>actual progeny #</b>	<b>observed ratio</b>
+/+	+/+	1/16	10	0.88/16
+/+	+/-	2/16	43	3.8/16
+/+	-/-	1/16	7	0.62/16
+/-	+/+	2/16	30	2.6/16
+/-	+/-	4/16	79	7.0/16
+/-	-/-	2/16	12	1.1/16
-/-	+/+	1/16	0	0/16
-/-	+/-	2/16	0	0/16
-/-	-/-	1/16	0	0/16
<b>Total</b>			181	



**Table 3**

PCR genotyping of embryos from intercrosses between *dab2*(+/-); *c-fos* (+/-) littermates

Stages	Litter #	Total Embryo #	(mean litter size)	Abnormal embryos <i>dab2</i> (-/-); <i>c-fos</i> (-/-)	ratio
E8.5	9	73	(8.1)	4	0.88/16
E9.5	8	59	(7.4)	3	0.81/16
E10.5	6	45	(7.5)	2	0.71/16
Pups	25	181	(7.2)	0	0/16

**Note:** The abnormal embryos were genotyped to be *dab2*; *c-fos* (-/-). The fraction of embryos expected to be *dab2* (-/-); *c-fos* (-/-) is 1/16.