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Disabled-2 is a FOXP3 target gene required for regulatory T cell function

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Abstract

FOXP3 expressing regulatory T cells are vital for maintaining peripheral T cell tolerance and homeostasis. The mechanisms by which FOXP3 target genes orchestrate context-dependent Treg cell function are largely unknown. Here we show that in mouse peripheral lymphocytes, the

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Drosophila Disabled-2 (*Dab2*) homolog, a gene that is involved in enhancing TGFβ responses, is exclusively expressed in FOXP3$^+$ regulatory T cells. *Dab2* is a direct target of FOXP3 and regulatory T cells lacking DAB2 are functionally impaired *in vitro* and *in vivo*. However, not all aspects of Treg cell function are perturbed and DAB2 appears dispensable for Treg cell function in maintaining naïve T cell homeostasis.

Introduction

FOXP3 expressing regulatory T (Treg) cells are a subset of suppressor CD4$^+$ T cells that are necessary for controlling peripheral T cell tolerance (1). Null mutations in the *Foxp3* gene in humans and mice leads to fatal, early onset autoimmunity (2). Treg cell mediated suppression *in vivo* is postulated to be highly context-dependent and likely involves failsafe, multiple effector components. The absolute requirement for FOXP3 in Treg cell function has stimulated intensive investigations to identify FOXP3 target genes and proteins. Phosphodiesterase 3b (*Pde3b*), which controls cAMP availability (3), *Ctla4*, which likely regulates APC function (4–6), *Ebi3*, which encodes for the IL-27β chain of regulatory cytokine, IL-35 (7), and microRNA *mir155* that maintains IL-2 responsiveness of Treg cells (8) are direct gene targets of FOXP3 that have been shown to be variably required for Treg cell function and homeostasis.

Here, we show that *Dab2* is a target gene of FOXP3 that is critical for Treg cell function *in vitro* and *in vivo*. Unlike previously identified targets of FOXP3, *Dab2* expression is restricted to CD4$^+$ Treg cells in peripheral lymphocyte subsets. In non-lymphoid cells, DAB2 has several critical functions in cell development and transformation by enhancing SMAD activation during TGFβ signaling (9,10), in GAP junction functions (11), and in clathrin coated pit-mediated endocytosis of cell surface receptors (12). *Dab2* expression is also regulated by the Vitamin A metabolite all-*trans* Retinoic Acid (ATRA) (13). Considering the role of TGFβ and ATRA in regulating FOXP3 expression in Treg cells as well as their function (14,15), we hypothesized that DAB2 is required for Treg cell function.

Analyses of mice with a T cell-restricted *Dab2*-deficiency showed that they generate normal numbers of Treg cells, but these Treg cells are not functional *in vitro*. While *Dab2*-deficient mice appear healthy and do not show significant perturbations in maintenance of peripheral naive T cell homeostasis, *Dab2*-deficient Treg cells are impaired in controlling colitogenic T cells in an adoptive transfer model.

Materials and Method

Mice

Dab2$^{fl/fl}$ (B6×129) mice (16) were backcrossed 4 times onto B6 background. T cell specific deletion of *Dab2* was obtained by crossing *Dab2*$^{fl/fl}$ mice with either *Cd2*CreTg$^+$ or *Cd4*CreTg$^+$ mice (17). *Foxp3*eGFP reporter mice (18) were a kind gift from V. Kuchroo (Harvard medical School). *Rag1*$^{-/-}$ and C57Bl/6 mice were obtained from Jackson Laboratories. All experiments were approved by the Umass Institutional Animal Care and Use Committee.

Antibodies, Flow cytometry and cell sorting

Cells were stained for surface markers (Abs from BD Bioscience and eBioscience) followed by FOXP3 staining according to eBioscience instructions. cAMP antibody was from Abcam. Samples were acquired on a Becton Dickinson LSRII flow cytometer and data analyzed using FlowJo software (Treestar). Thymic and peripheral T cell subsets were sorted to greater than 95% purity using using a MoFlo cell sorter (Dako).

Retroviral Infection

NFC (αβ, $CD4^+CD8^+$) thymoma cells were infected with a control MSCV retroviral vector containing GFP or with an MSCV retroviral vector carrying the full-length *Foxp3*cDNA cloned upstream of an IRES and GFP. Stable cell lines that express vector alone (V) and MSCV-*Foxp3* (F) were generated by cell sorting and maintained in complete DMEM (10% FBS, 50μM 2-mercaptoethanol, 2mM L-glutamine, 20mM Hepes, 0.1mM non-essential amino acids).

RT-PCR and real-time PCR

RNA was isolated from cells with Trizol Reagent (Invitrogen) and cDNA was prepared using an Omniscript RT-PCR kit (Qiagen). For semi-quantitative RT-PCR (sqRT-PCR), 3-fold serial dilutions of cDNA were used. PCR primers are listed in Supplemental Data 1. Real-time PCR amplification was performed using iQ SYBR Green supermix (BioRad). All data were normalized to *Actb* mRNA expression and represented as arbitrary units (AU).

Chromatin Immunoprecipitation (ChIP) Assays

FOXP3 over-expressing NFC cells (F) and vector control cells (V) were stimulated with PMA (50ngml) and ionomycin (100ng/ml) at 37°C for 24 hours. ChIP assays were performed on 1×10^6 cells using the ChIP Assay Kit (Upstate Cell signaling Solutions). Immuno-precipitation was performed using anti-FOXP3 antibody (Santa Cruz). The recovered DNA was dissolved in 20 μl of H_2O and analyzed by PCR. PCR primers used are listed in Supplemental Figure 1.

In vitro FOXP3 induction

Sorted $CD4^+FOXP3^-$ T cells from *Foxp3*eGFP reporter mice were activated with plate-bound anti-CD3 (0.5μg/ml; clone 500A2) and anti-CD28 (1.0μg/ml; clone 37N) in the presence of rTGFb (2ng/ml; R&D) and all-trans retinoic acid (ATRA) (100nM; Sigma).

In vitro suppression assay

Sorted $CD4^+CD25^+$ were activated *in vitro* as described above, in the presence of 100U/ml of rIL-2 in complete DMEM for 48 hours. Post-stimulation, indicated numbers of Treg cells were co-cultured with 5×10^4 freshly isolated $CD4^+$T cells and 5×10^4 irradiated splenocytes from B6 mice and activated with plate-bound anti-CD3 for 72 hours. [^{3}H]TdR incorporation was measured over the last 6 hours.

Colitis induction and cure

4×10^5 naïve $CD4^+CD25^-$ cells from *wt* mice and, 2×10^5 sorted $CD4^+CD25^+$ regulatory T cells were co-injected into lymphocyte-deficient (*Rag1*$^{-/-}$) mice. For colitis “rescue” experiments, $4x10^5$ naïve $CD4^+CD25^-CD45RB^{hi}$ T cells were injected into *Rag1*$^{-/-}$ mice to induce colitis. 4 weeks later, $1x10^6$ $CD4^+CD25^+$ regulatory T cells were injected. Colonic pathology was determined at 2–3 weeks after transfer of Treg cells by standard H&E staining of formalin fixed tissues. Colitis was scored as described in (19).

Calcein AM intercellular transfer, cAMP ELISA

Sorted $CD4^+CD25^+$ Treg cells were loaded with calcein as described in (20) and co-cultured with $Ly5.1^+CD4^+$T cells. Cells were activated as described above and calcein transfer was detected by flow cytometry after 16 hours. To measure intracellular cAMP amounts, sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were washed in ice-cold PBS and lysed in 0.1NHCl and a cAMP specific ELISA was performed according to the manufacturer’s protocol (BioMol and Promega).

Results and Discussion

Dab2 expression is restricted to FOXP3$^+$ Treg cells

We infected NFC αβTCR+ CD4$^+$CD8$^+$ cells with a retrovirus vector containing *Foxp3* cDNA, followed by global gene expression profiling to determine genes whose expression was changed by the ectopic *Foxp3* expression (*data not shown*). One of only a few genes that were altered in expression was *Dab2* (Drosophila Disabled homolog-2), as illustrated by a confirmatory RT-PCR assay (Fig. 1A). In peripheral T cell subsets, *Dab2* expression was restricted exclusively to CD4$^+$FOXP3$^+$ Treg cells (Fig. 1B). In the thymus, *Dab2* was expressed in early precursor cells (*data not shown*) and in mature FOXP3$^+$ CD4SP (CD4$^+$CD8$^-$) cells (Fig. 1C). *Dab2* was also expressed in TGFβ-induced FOXP3$^+$ Treg cells *in vitro* and its expression was enhanced by ATRA (Fig. 1D), an inducer of *Dab2* expression in non-lymphoid cells.

To determine whether FOXP3 can bind to the *Dab2* regulatory region, we performed ChIP analysis by selecting relevant FOXP3 consensus binding sites in the regulatory sequences of *Dab2* based on conservation between the mouse and human *Dab2* genomic loci. Among four such conserved consensus binding sites tested, only one located in the 5' untranslated region, ~2.3 kb upstream of the transcriptional site of the *Dab2* gene, was found to be associated with FOXP3 in FOXP3$^+$ NFC cells (Fig. 1E). These results identify *Dab2* as a potential direct target gene of FOXP3.

Dab2 deficiency alters Treg cell function

To determine the function of DAB2 in Treg cells, we generated mice lacking *Dab2* specifically in T cells. We analyzed both *Cd2* promoter-Cre Tg$^+$×*Dab2*$^{fl/fl}$ and *Cd4* promoter-Cre Tg$^+$×*Dab2*$^{fl/fl}$ mice that delete the floxed *Dab2* gene during early (CD3$^-$ CD4$^-$CD8$^-$ TN) and late (CD4$^+$CD8$^+$ DP) stages of intrathymic T cell development, respectively. Treg cells in conditional *Dab2*-knockout (CKO) mouse lines lacked *Dab2* expression (Fig. 1B). As Treg cells in both models were indistinguishable in function and phenotype we present data predominantly from the analysis of *Dab2*$^{fl/fl}$*:Cd4* mice, herewith referred to as *Dab2* CKO mice.

Mice lacking DAB2 in Treg cells were healthy in appearance and did not suffer from overt autoimmunity even at 10–12 months of age, suggesting that the mutant Treg cells were capable of maintaining naïve T cell tolerance. The frequency of thymic and peripheral CD4$^+$FOXP3$^+$ cells was unchanged in *Dab2* CKO mice compared to *wt* mice. The Treg cells were also phenotypically similar to *wt* Treg cells and expressed normal levels of CTLA-4, GITR and IL-7R (Supplemental Figure 2).

We first tested the functionality of *Dab2* CKO Treg cells in an *in vitro* suppression assay. Whereas *wt* Treg cells suppressed the proliferation of responder T cells in a Treg cell number-dependent manner, Treg cells from *Dab2* CKO mice were unable to do so (Fig 2C). Next, we determined the ability of purified Treg cells from *Dab2* CKO mice to control co-injected pathogenic naïve (CD4$^+$CD25$^-$) T cells when transferred into lymphopenic animals. In contrast to the complete lack of function of *Dab2*-deficient Treg cells *in vitro*, they were as effective as *wt* Treg cells in preventing the induction of colitis by naïve T cells transferred into *Rag1*$^{-/-}$ mice (Fig. 2D). However, in a more stringent model of Treg cell function, *Dab2*-deficient Treg cells failed to moderate established colitis (21) (Fig. 2E). While *wt* Treg cells effectively cured colitis, *Dab2* CKO Treg cells were defective in regulating on-going, aggressive lymphocyte infiltration and accumulation in the colon that led to a progressive wasting disease (Fig. 2F).

Impaired Gap-junction function by Dab2-deficient Treg cells

We next investigated the mechanistic basis for the observed defects in *Dab2*-deficient Treg cells by examining potential molecules and/or pathways involved in Treg cell-mediated immune suppression. Treg cells from *Dab2*-deficient mice produced normal amounts of IL-10, expressed comparable amounts of *Il10*, *TGFβ* and *Ebi3* at the transcriptional level and expressed normal amounts of Granzyme B (*data not shown*). One mechanism by which Treg cells suppress target cell proliferation and activation is by transferring cAMP through GAP junctions to effector T cells (20). Since DAB2 is known to interact with connexins that make up the GAP junctions (11), we tested whether *Dab2*-deficient Treg cells were poor suppressors *in vitro* because they had deficiencies in GAP junction-mediated intercellular communication (GJIC). We labeled Treg cells from *wt* and *Dab2* CKO mice with a GAP junction transferable dye, Calcein AM, and co-cultured these with congenically marked *wt* responder cells. *Dab2*-deficient Treg cells were unable to transfer the dye as efficiently as *wt* Treg cells (Fig. 3A). In addition, *Dab2*-deficient Treg cells expressed higher amounts of intracellular cAMP compared to *wt* Treg cells (Fig. 3B,C). These results suggest that one impaired function in *Dab2*-deficient Treg cells involves the GJIC, while other mechanisms of Treg cell-mediated suppression examined appeared largely intact.

Collectively, we have identified *Dab2* as a FOXP3 target gene that is required for a subset of Treg cell function. *Dab2* expression is higher in $CD44^+$ Treg cells compared to $CD44^-$ Treg cells (*data not shown*) and whether all $FOXP3^+$ Treg cells express *Dab2* is currently under investigation. Although *Dab2* is expressed only in Treg cells among peripheral lymphocytes, it is also expressed specifically in TN3 thymocytes and is required for normal TGFβ and/or Activin responses of lymphocytes (Jain et al., manuscript in preparation).

Given the FOXP3-dependent expression of *Dab2* in mature Treg cells and the established function of DAB2 in promoting TGFβ signaling (9), one possibility was that *Dab2* CKO mice would phenocopy the loss of Treg cells observed in mice with defective TGFβ signaling (22). However, *Dab2* CKO mice maintained normal numbers of Treg cells, suggesting that DAB2 may be required for amplification, rather than maintenance, of TGFβ signaling in as yet undefined context. *Dab2*-deficient Treg cells do not function *in vitro* and cannot cure established colitis *in vivo*, but they retain sufficient suppressive activity to maintain T cell homeostasis in unperturbed mice. This selective Treg cell defect is another indication that Treg cells employ multiple, context-dependent modes of immunosuppression. Although the reduced GAP junction activities appear to be the only distinguishing feature of *Dab2*-deficient Treg cells that has previously been correlated with impaired *in vitro* suppression, whether this is the cause of impaired function of *Dab2*-deficient Treg cells remains to be established. The *in vivo* context in which DAB2 is essential for Treg cell function is an outstanding issue that will require further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

Treg	T regulatory
ATRA	all-trans retinoic acid
CKO	conditional knock-out

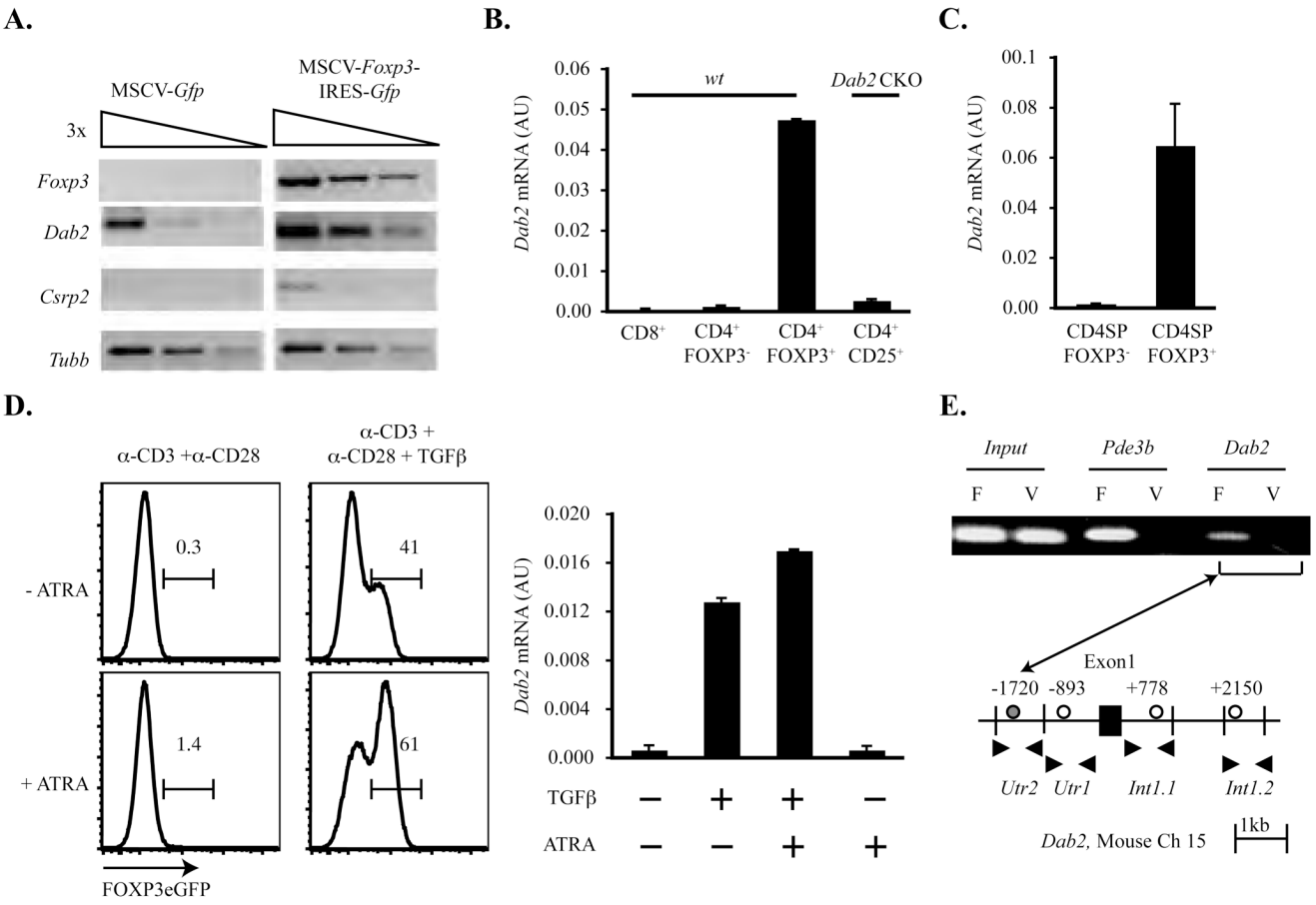

Figure 1. FOXP3 dependent *Dab2* expression in regulatory T cells

A. Semi-quantitative RT-PCR (sqRT-PCR) analysis of *Dab2* expression in NFC cells infected with a control retroviral vector (MSCV-*Gfp*) and a retroviral vector expressing *Foxp3* cDNA (MSCV-*Foxp3*-IRES-*Gfp*). *Csrp2*, Cysteine-rich protein 2, is a potential TGFβ-regulated gene that has been classified as a Treg cell signature gene (23). **B.** Real-time PCR analysis of *Dab2* expression in FOXP3$^+$ cells sorted from *Foxp3–gfp* mice based on GFP expression and from *Dab2* CKO mice based on CD25 expression. **C.** Real-time PCR analysis of *Dab2* expression in thymic CD4SP (CD4$^+$CD8$^-$) FOXP3$^-$ and CD4SPFOXP3$^+$ cells sorted from *Foxp3–gfp* mice. Data in **B** and **C** are normalized to *Actb* mRNA expression and are representative of 3 individual experiments. *AU*: Arbitrary Units **D.** (*left panel*) FACS histograms showing FOXP3-GFP expression in sorted CD4$^+$GFP$^-$ naïve T cells stimulated with anti-CD3 and anti-CD28 in the presence or absence of TGFβ (2ng/ml) and ATRA (100nM); (*right panel*) Real-time PCR analysis of *Dab2* mRNA expression, normalized to *Actb* mRNA, in each stimulation condition. Data are representative of 3 individual experiments. *AU*: Arbitrary Units **E.** 4 FOXP3 consensus binding sites were identified in the *Dab2* gene regulatory element (circles), two upstream of exon 1 (*Utr1*, *Utr2*) and two in intron 1 (*Int1.1*, *Int1.2*). ChIP assay (one of two independent experiments) on PMA/Ionomycin activated NFC-Foxp3 (F) and NFC-vector alone (V) cells using anti-FOXP3 Ab showed FOXP3 binding only to *Utr2* region (filled circle). Anti-FOXP3 antibody binding to *Pde3b* locus (3) was used as a positive control.

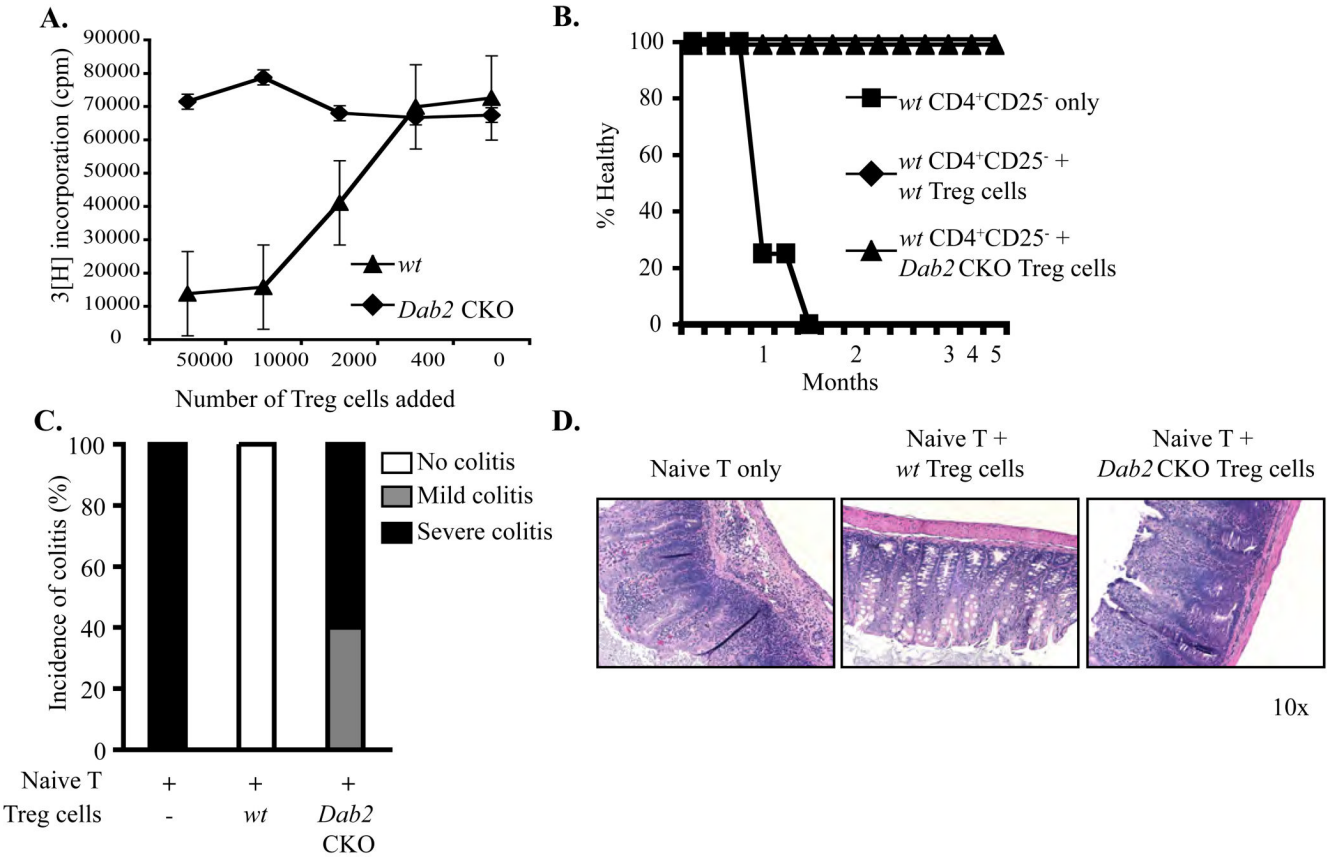

Figure 2. *Dab2* deficient Treg cells are functionally impaired

A. Proliferation of responder $CD4^+$ effector T cells co-cultured with varying numbers of sorted $CD4^+CD25^+$ cells from *wt* or *Dab2* CKO mice and stimulated with plate bound anti-CD3 plus anti-CD28. One of six independent experiments with similar results is shown. **B.** *Rag1*$^{-/-}$ mice were injected with $CD4^+CD25^-$ T cells alone or co-injected with $CD4^+CD25^+$ cells from *wt* or *Dab2* CKO mice (n=4 for each group; one of three independent experiments is shown). Mice were weighed and health was monitored weekly for visible signs of colitis, inflammation or wasting. Mice were scored as unhealthy at the first signs of inflammation or weight loss. **C.** Naïve $CD4^+CD25^-$ $CD45RB^{hi}$ T cells were injected into *Rag1*$^{-/-}$ recipients. 4 weeks after transfer, mice received $CD4^+CD25^+$ Treg cells from *wt* and *Dab2* CKO mice. Colitis was scored as described in (19). **D.** At 2–3 weeks after transfer of Treg cells, mice were euthanized and colons were prepared for histological analysis. Representative H&E stained sections of colons are shown. Original magnification is 10x.

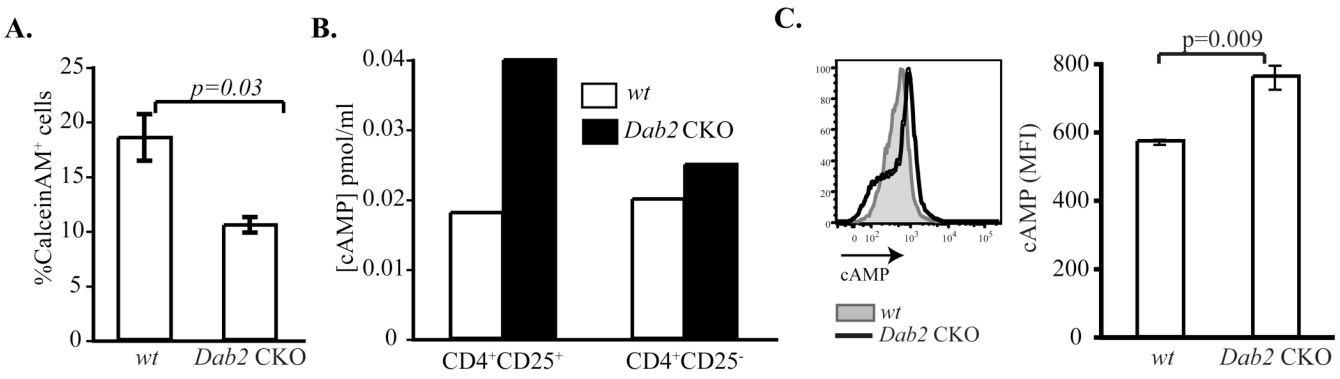

Figure 3. *Dab2* deficient Treg cells have impaired Gap-junction function

A. $CD4^+$T cells from Ly5.1 congenic mice were co-cultured with Calcein AM-loaded *wt* or *Dab2* CKO $CD4^+CD25^+$ Treg cells and stimulated with plate-bound anti-CD3 plus anti-CD28. The frequency of $Calcein^+Ly5.1^+$ cells at 16 hours of culture was determined. Data are averaged over 3 independent experiments. **B.** Cytosolic cAMP concentrations in sorted $CD4^+CD25^+$ Treg cells and $CD4^+$ $CD25^-$ conventional T cells from *wt* and *Dab2* CKO mice as measured by a standard cAMP ELISA. One of two independent experiments with similar results is shown. **C.** (*Left*) Representative flow cytometric plot of intracellular staining of cAMP in *ex vivo wt* and *Dab2* CKO $CD4^+FOXP3^+$ cells. (*Right*) Mean fluorescence intensity (MFI) of cAMP staining in $CD4^+FOXP3^+$ cells. Data are a mean of 2 independent experiments with at least 6 mice in each group. Error bars are standard error of means. P-values were calculated using Student's t-test.