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Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development

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SUMMARY

Plasmacytoid dendritic cells (PDCs) represent a unique immune cell type specialized in type I interferon (IFN) secretion in response to viral nucleic acids. The molecular control of PDC lineage specification has been poorly understood. We report that basic helix-loop-helix transcription factor (E protein) E2-2/Tcf4 is preferentially expressed in murine and human PDCs. Constitutive or inducible deletion of murine E2-2 blocked the development of PDCs but not of other lineages and abolished IFN response to unmethylated DNA. Moreover, E2-2 haploinsufficiency in mice and in human Pitt-Hopkins syndrome patients was associated with aberrant expression profile and impaired IFN response of the PDC. E2-2 directly activated multiple PDC-enriched genes, including transcription factors involved in PDC development (SpiB, Irf8) and function (Irf7). These results identify E2-2 as a specific transcriptional regulator of the PDC lineage in mice and humans and reveal a key function of E proteins in the innate immune system.

INTRODUCTION

Dendritic cells (DCs) play critical roles in immunity because of their ability to recognize invading pathogens and mobilize multiple immune cell types to combat them. Conventional DCs (cDCs) efficiently detect and present foreign antigens to antigen-specific T lymphocytes in the context of major histocompatibility complex (MHC) molecules. On the other hand, plasmacytoid DCs (PDCs) represent a distinct DC type specialized in rapid secretion of type I interferons (interferon α [IFNα] and IFNβ) in response to viruses (Asselin-Paturel and Trinchieri, 2005; Barchet et al., 2005; Cao and Liu, 2007). The resulting IFN acts both directly to block viral replication and as an adjuvant to activate multiple immune cell types. In particular, PDCs efficiently suppress HIV replication, and their infection and eventual depletion contributes to immunodeficiency caused by HIV (Meyers et al., 2007). Conversely, persistent activation of PDCs causes elevated IFN levels in autoimmune diseases such as lupus and psoriasis (Banchereau and Pascual, 2006). In the latter condition, aberrant activation of PDCs by complexes of self-DNA has been recently demonstrated (Lande et al., 2007). Thus, PDCs are primary intereferon-producing cells that play central roles both in protective antiviral responses and in immunopathology.

PDCs express a combination of Toll-like receptors (TLRs) including TLR7/8 and TLR9, allowing the recognition of virus-associated nucleic acids such as single-stranded RNA and unmethylated CpG-containing DNA (CpG), respectively. After TLR-mediated virus recognition, PDCs produce IFN and other cytokines and subsequently differentiate into activated cDCs. The secretion of IFN by PDCs is characterized by rapid kinetics, high level (up to 1000-fold higher than most cell types), and broad spectrum of IFN types (α and β). This is facilitated by multiple mechanisms, including high secretory capacity reflected in "plasmacytoid" (i.e., plasma cell-like) morphology; high basal expression of IRF7, the key transcriptional regulator of IFN response (Barchet et al., 2002); and prolonged retention of TLR ligands in early endosomes (Honda et al., 2005). In addition, PDCs specifically express several unique receptors that modulate IFN production, including human BDCA-2/CD303 and ILT7 and murine SiglecH (Gilliet et al., 2008). Thus, the unique functional

properties of PDCs are reflected in their specific gene expression program.

Although PDCs clearly represent a distinct hematopoietic cell lineage, the molecular and cellular basis of their development is poorly understood. PDCs develop in the bone marrow (BM) from a common progenitor of both cDCs and PDCs (also termed "pro-DCs" [Naik et al., 2007; Onai et al., 2007]). On the other hand, the developmental progression between pro-DC and committed PDC remains uncharacterized (Wu and Liu, 2007). An important signal for PDC development is provided by cytokine Flt3 ligand (Flt3L) through its receptor Flt3 and transcriptional effector Stat3; however, the same molecules also drive the development of cDCs (Laouar et al., 2003; Onai et al., 2006). Moreover, Stat3 mediates the proliferation of cDC and PDC progenitors but is dispensable for the specification of PDCs (Esashi et al., 2008). Transcription factor Irf8 is required for PDC development (Schiavoni et al., 2002; Tsujimura et al., 2003); at the same time, it is essential for the development and/or function of macrophages and cDCs (Wu and Liu, 2007). Transcription factor SpiB is preferentially expressed in the PDC, and its RNAi-mediated knockdown specifically impaired human PDC development in vitro (Schotte et al., 2004); however, the role of SpiB in PDC development in vivo is unknown. Thus, additional transcriptional mechanisms are likely to specify the unique identity and expression profile of the PDC.

E proteins comprise a family of basic helix-loop-helix (bHLH) transcription factors homologous to *Drosophila* protein Daughterless (Lazorchak et al., 2005; Murre, 2005). E proteins include E12 and E47 (encoded by a single gene, *E2a*), HEB, and E2-2, which form homodimers or heterodimers with other family members. E protein dimers bind E box sequences (CANNTG) with an apparent preference for C or G in the middle positions. The activity of E proteins is antagonized by the Id proteins (Id1–Id4), which sequester them into nonfunctional heterodimers. The *E2a* gene products are absolutely required for B lymphocyte development and maintenance, including immunoglobulin gene rearrangement and the expression of key genes such as *RAG1/2* and *Tdt* (*Dntt*). Similarly, a combined activity of E2a and HEB regulates T cell receptor rearrangement, proliferation, and selection in developing T lymphocytes. In contrast, E2-2-deficient lymphocytes develop normally, showing a slight reduction only in competitive settings (Bergqvist et al., 2000; Wikstrom et al., 2006; Zhuang et al., 1996). Thus, E2-2 is largely dispensable for T and B lymphocyte development, and its function in the immune system has remained unclear.

Specification of several cDC subsets requires the expression of E protein inhibitors such as Id2; in contrast, PDCs are not affected by the loss of Id2 (Hacker et al., 2003). Conversely, overexpression of Id proteins in human hematopoietic progenitors inhibited the development of both T and B lymphocytes and PDCs but not of cDCs (Spits et al., 2000). Furthermore, mature PDCs express genes that represent E protein targets in early lymphocyte development, such as *RAG1/2* genes, *TdT* (*Dntt*), *VpreB*, and pre-T cell receptor α (*PTCRA*) (Harman et al., 2006; Pelayo et al., 2005; Shigematsu et al., 2004). These observations prompted us to analyze the expression and function of E proteins in the PDC lineage. We now report that E protein E2-2 is a specific regulator of PDC development and of PDC-dependent IFN responses in mice and in humans.

RESULTS

E2-2 Is Preferentially Expressed in the PDC

The analysis of E protein gene expression in sorted murine PDCs by quantitative RT-PCR (qRT-PCR) showed that *E2a* (*Tcfe2a*) and *HEB* (*Tcf12*) were expressed comparably in the PDC and in other cell types (Figure 1A). On the other hand, *E2-2* (gene symbol *Tcf4*; thereafter called *E2-2* to avoid confusion with Tcf/LEF family member *Tcf4/Tcf7l2*) was abundantly expressed in PDCs, expressed at lower levels in B cells, and barely detectable in other cell types, a pattern similar to that of *SpiB*. The same PDC-enriched expression of *E2-2* was observed in the human PDC (Figure 1B). Conversely, E protein inhibitor *Id2* was abundantly expressed in cDCs but excluded from murine PDCs, likely facilitating E protein activity in the latter (Figure 1C). Furthermore, the expression of *E2-2* as well as of *SpiB* was reduced after CpG-induced activation of PDCs, consistent with the ongoing differentiation into cDCs (Figure 1D).

The analysis of E protein expression in the human PDC cell line CAL-1 (Maeda et al., 2005) showed that E2-2 protein was detected in CAL-1 but not in T cell lymphoma line MOLT-4 (Figure 1E). Indeed, genome-wide expression analysis revealed that *E2-2*, along with *SPIB*, *IRF8*, and *IRF7*, is highly enriched in CAL-1 compared to MOLT-4 (Figure S1 and Document S2 available online). Electrophoretic mobility shift assay (EMSA) showed that E box-binding nuclear protein complexes in CAL-1 were supershifted by antibodies to E2-2 but not to E2A, whereas the opposite was observed in MOLT-4 (Figure 1F). Similar to murine PDCs, CpG-treated CAL-1 cells showed reduced E2-2 expression and low levels of E2-2-containing E protein complexes (Figure S2). These data demonstrate that E2-2 is expressed preferentially in the mature, steady-state PDCs and is a major component of E box-binding protein complexes in these cells.

The Development of *E2-2*$^{-/-}$ PDCs Is Blocked at an Immature Stage

To test the role of E2-2 in PDC development, we analyzed mice with a germline mutation of *E2-2* (Zhuang et al., 1996). Because *E2-2*$^{-/-}$ knockout (KO) animals die at birth, we transferred *E2-2*$^{+/+}$ control or *E2-2*$^{-/-}$ KO fetal liver cells (expressing the CD45.2 allelic marker) into lethally irradiated CD45.1 recipients and analyzed the development of donor-derived CD45.2^{+} hematopoietic cells. Murine PDCs are CD11c^{low}, Ly-6C^{+}, B220^{+} and express specific markers such as Bst2 (mPDCA1) and SiglecH. We found that KO fetal liver cells mediated efficient hematopoietic reconstitution and gave rise to myeloid cells, T and B lymphocytes, and CD11b^{+} (CD8^{-}) and CD11b^{-} (CD8^{+}) cDC subsets (Figure 2A and Figure S3). In contrast, KO-derived PDCs were completely absent from the BM and all lymphoid organs (Figures 2A and 2B and Figure S4). Consistent with the absence of PDCs, no IFNα could be detected in the supernatants of KO donor-derived splenocytes and BM cells after culture with type A CpG (n = 3; data not shown). Thus, the deletion of E2-2 in hematopoietic stem cells completely blocks the emergence of PDCs.

In agreement with the blocked PDC development in vivo, Flt3L-supplemented cultures of KO-reconstituted BM failed to

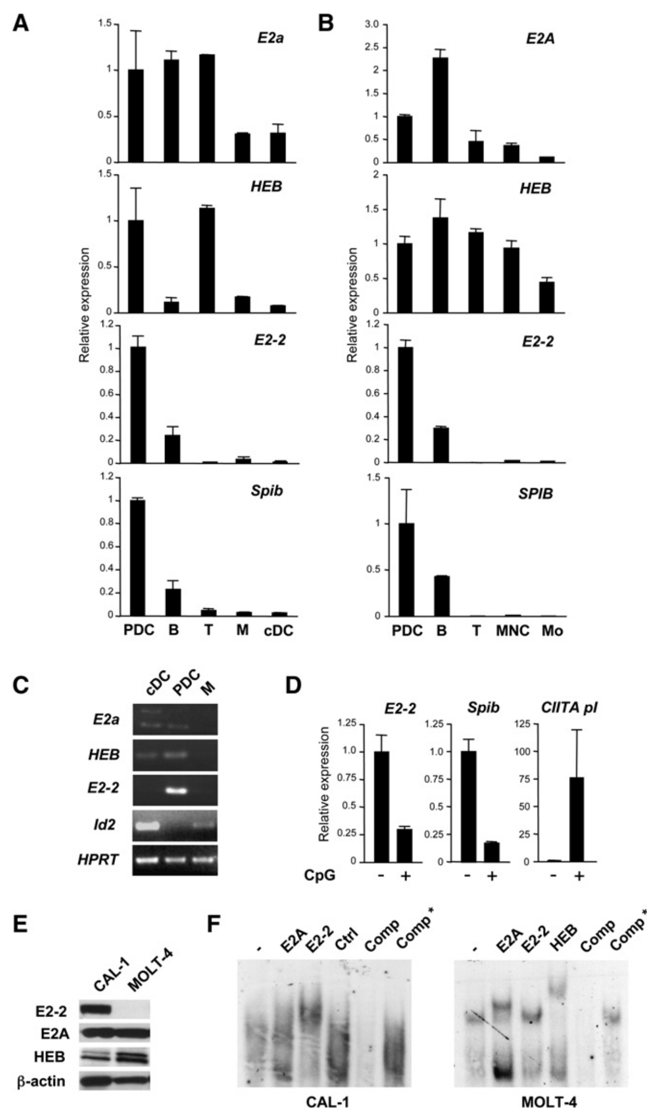

Figure 1. E2-2 Is Preferentially Expressed in the PDC

(A) Expression of E protein genes in sorted splenic cell populations from wild-type mice, including PDCs (CD11c^{low} Bst2^{+}), B cells (CD11c^{-} B220^{+}), T cells (TCRβ^{+}), monocytes and macrophages (M, CD11c^{-} CD11b^{+} side scatter^{low}), and cDCs (CD11c^{high} CD11b^{+}). Data represent normalized expression levels relative to the PDC sample, as determined by qRT-PCR (mean ± SD of triplicate reactions). The expression of the PDC-enriched transcription factor *SpiB* is shown as a control.

(B) Expression of E protein genes in sorted human peripheral blood cell populations, determined by qRT-PCR as above. Cells include PDCs (BDCA-4^{+}), B cells (CD19^{+}), T cells (CD3^{+}), monocytes (Mo, CD14^{+}) and total mononuclear cells (MNC).

(C) The expression of E protein genes and *Id2* in PDCs. Shown is RT-PCR analysis of cell populations sorted as described in (A).

(D) The expression of *E2-2* in PDCs undergoing differentiation into cDCs. BM culture-derived PDCs were purified, incubated with type B CpG for 48 hr (+), and analyzed by qRT-PCR for the expression of *E2-2*, *SpiB*, and the cDC-specific *pI* transcript of class II transactivator (*CIITA pI*) as a control. Data represent normalized expression levels relative to the untreated (–) PDC sample (mean ± SD of triplicate reactions).

(E) The expression of E proteins in the human PDC cell line. Shown is Western blot analysis of the indicated proteins in the PDC line CAL-1 and in T cell lymphoma MOLT-4.

(F) The analysis of E box-binding nuclear proteins in the PDC. Shown is EMSA with radiolabeled E box-containing probe and nuclear extracts from CAL-1 or MOLT-4 cells. The binding reactions contained no additives (–), control (Ctrl) or E protein-specific antibodies, unlabeled probe as a competitor (Comp), or unlabeled probe with a mutated E box (Comp*). No supershift was observed with anti-HEB antibody in CAL-1 (data not shown).

generate CD11c^{+} B220^{+} PDCs and consisted entirely of CD11c^{+} CD11b^{+} cDCs (Figure 2C). These cultures showed the initial presence of donor-derived CD11c^{+} CD11b^{-} B220$^{-/low}$ population, suggesting that E2-2-deficient progenitors initiated development toward the PDC lineage but failed to differentiate. To test this notion in vivo, we analyzed donor-derived BM cells in control- and KO-reconstituted chimeric mice (Figure 2D). In control BM, nearly all donor-derived CD11c^{+} CD11b^{-} cells were Flt3^{low} and Ly-6C^{+}, and the majority expressed PDC markers B220 and Bst2; some cells also expressed peripheral PDC marker Ly-49Q. Notably, a small fraction of CD11c^{+} CD11b^{-} cells negative for B220 or Bst2 was also present in control BM. In KO BM, the reduced CD11c^{+} CD11b^{-} population expressed Flt3 and Ly-6C, but was low or negative for B220, Bst2, and Ly-49Q. Thus, E2-2-deficient BM PDCs accumulated as an immature CD11c^{+} Ly-6C^{+} Bst2^{-} population, which likely represents a natural early stage of PDC differentiation. Indeed, lineage tracing with DC- and PDC-specific *CD11c*-Cre strain indicates that this population has initiated development along the PDC lineage (Figure S5). These data suggest a developmental sequence from the CD11c^{-} pro-DC to immature CD11c^{+} Ly-6C^{+} PDC to mature CD11c^{+} Ly-C6^{+} Bst2^{+} B220^{+} PDC, with the latter maturation step being fully dependent on E2-2 expression (Figure 2E).

Conditional Inactivation of E2-2 Impairs PDC Development

To test the consequences of E2-2 inactivation in the adult organism, we used a conditional LoxP-flanked ("floxed") allele of *E2-2* (Bergqvist et al., 2000) crossed to *R26*-CreER mice, which ubiquitously express a tamoxifen-inducible Cre recombinase (CreER). Conditional knockout (CKO, *E2-2*$^{flox/flox}$, *R26*-CreER^{+}) mice and Cre-expressing wild-type controls (*E2-2*$^{wt/wt}$, *R26*-Cre^{+}) were analyzed 10 days after initial tamoxifen administration, at which point efficient *E2-2* combination was observed in CKO spleens (Figure S6). Compared to controls, the absolute numbers of PDCs in the BM and spleens of CKO mice were reduced ~7-fold and >10-fold, respectively (Figure 3A). In contrast, total CKO splenocyte numbers were only marginally reduced (1.6-fold), and the relative content of all major cell types was unchanged. Furthermore, the BM of CKO animals failed to give rise to PDCs in Flt3L cultures in vitro (Figure 3B). Although control total spleen and BM cells manifested robust IFN secretion in response to CpG, the cells from CKO mice failed to produce IFN (Figure 3C). Thus, widespread loss of E2-2 selectively abolishes PDC development during adult hematopoiesis and eliminates PDC-dependent IFN response.

To test for a potential role of other E proteins in the PDC lineage, we used the same *R26*-CreER system to induce a simultaneous loss of both E2a and HEB (Jones and Zhuang, 2007). No

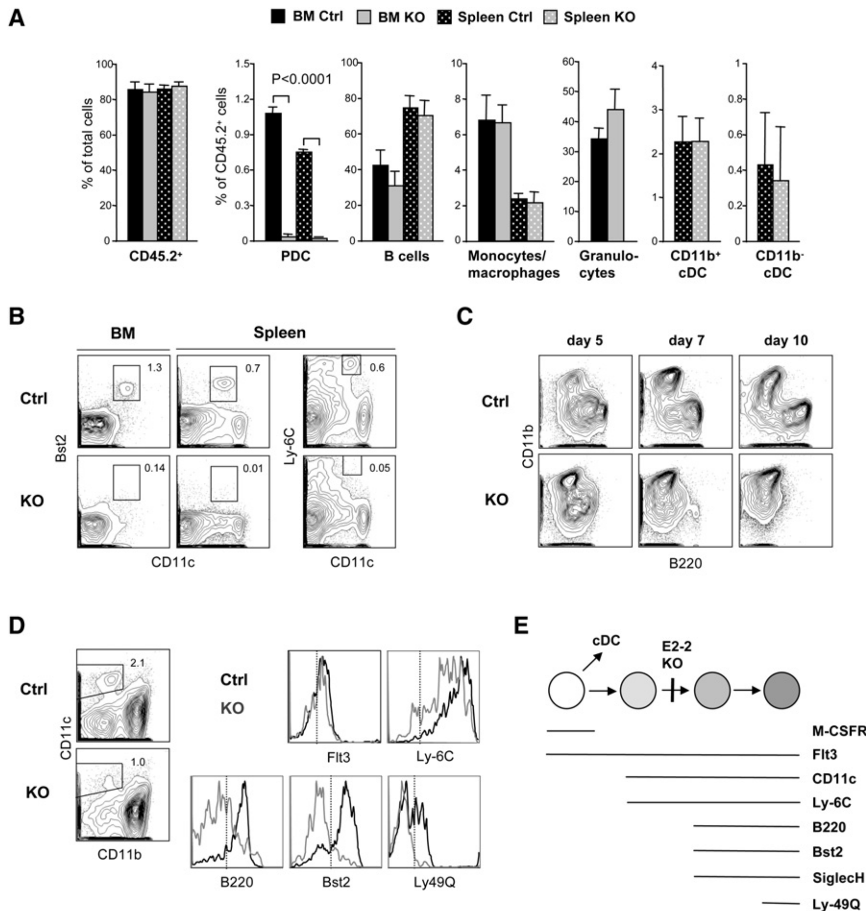

Figure 2. E2-2 Deficiency Blocks PDC Development

(A) The analysis of hematopoietic chimeras reconstituted with $E2\text{-}2^{+/+}$ control (Ctrl) or $E2\text{-}2^{-/-}$ knockout (KO) fetal liver cells. Shown are the fraction of donor-derived ($CD45.2^+$) cells in the total BM and spleen and the fractions of indicated cell types among the donor-derived $CD45.2^+$ cells. Cells included PDCs ($CD11c^{low}$ $Bst2^+$), B cells ($CD11c^-$ $B220^+$), monocytes and macrophages ($CD11c^-$ $CD11b^+$ side $scatter^{low}$), granulocytes ($CD11b^+$ side $scatter^{high}$), and cDCs ($CD11c^{high}$ MHC cl. II^+ $CD11b^+$ or $CD11b^-$). Data represent mean percentage ± SD of three control and five KO chimeras.

(B) Representative staining profiles of donor-derived ($CD45.2^+$) spleen and BM cells, with the PDC population indicated.

(C) PDC development in Flt3L-supplemented BM cultures from control or E2-2 KO-reconstituted chimeric mice. Shown are representative staining profiles of donor-derived $CD11c^+$ cells at the indicated days of culture.

(D) The analysis of PDC population in the BM of control or E2-2 KO-reconstituted chimeric mice. The left panel shows representative staining profiles of $CD45.2^+$ total BM cells, with the PDC-containing $CD11c^+$ $CD11b^-$ population indicated. The right panel shows fluorescence histograms of $CD45.2^+$ $CD11c^+$ $CD11b^-$ cells stained for the indicated surface markers; dotted line indicates positive staining threshold.

(E) A model of sequential PDC development in the mouse BM, showing pro-DCs (open circle), immature PDCs (light gray), and mature lineage marker-positive PDCs (dark gray). Immature PDCs were low or negative for M-CSFR and SiglecH (data not shown). The developmental block caused by E2-2 deletion is indicated.

reduction of PDCs was observed in the BM or spleen of induced E2a/HEB CKO animals, suggesting that both genes are largely dispensable for PDC development (Figure 3D). On the other hand, E2a/HEB CKO but not E2-2 CKO animals showed a profound dysregulation of splenic follicular B cells, consistent with a key role of E2a in this B cell subset (Quong et al., 2004). These data show that E2-2 does not play a major role in splenic B cells but is required for PDC development independently of other E proteins.

To confirm the PDC-intrinsic function of E2-2, we crossed $E2\text{-}2^{flox}$ mice to the *CD11c*-Cre deleter strain, in which Cre recombination is nearly complete in cDCs and is significant but incomplete in the PDC (60%–80%) but minimal in other lineages (Caton et al., 2007). Consistent with the expected recombination frequency, the PDC population showed a significant ~3-fold reduction in the BM of $E2\text{-}2^{flox/flox}$, *CD11c*-Cre^+ CKO mice compared to $E2\text{-}2^{wt/wt}$, *CD11c*-Cre^+ controls (Figure 3E). The splenic PDC population showed a smaller ~2-fold reduction, possibly due to expansion or influx of nondeleted cells. In contrast, the fraction and subset distribution of splenic cDCs were not affected. Altogether, conditional targeting of E2-2 demonstrates its essential cell-intrinsic role in PDC development.

E2-2 Is Haploinsufficient for PDC Development

To test whether the reduced levels of E2-2 might affect the PDC population, we analyzed heterozygous $E2\text{-}2^{+/-}$ animals. The number of PDCs in the BM and spleen was significantly decreased in these mice (Figures 4A and 4B); furthermore, Flt3L-supplemented cultures of $E2\text{-}2^{+/-}$ BM yielded a low fraction of PDCs (Figure 4C). In addition, $E2\text{-}2^{+/-}$ PDCs manifested an abnormal surface staining profile, including reduced expression of CD4, Thy1, and Ccr9 and increased levels of CD8 and CD11c (Figure S7). In contrast, $E2\text{-}2^{+/-}$ animals contained normal numbers of all splenic cell types including cDCs and B cells (Figure 4B), and showed normal B cell development in the BM and spleen (Figure S8). Thus, E2-2 heterozygosity causes impaired development and abnormal phenotype of the PDC.

To test the function of $E2\text{-}2^{+/-}$ PDCs, we used a mouse reporter strain expressing enhanced yellow fluorescent protein (EYFP) marker from the endogenous *IFNβ* locus. As described for *IFNα* reporter mice (Kumagai et al., 2007), the injection of lipid-complexed type A CpG into *IFNβ*-EYFP mice induced EYFP expression exclusively in the PDC (S.S. and R.L., unpublished data). After in vivo challenge with CpG, the intensity of EYFP fluorescence peak was decreased in the PDCs from

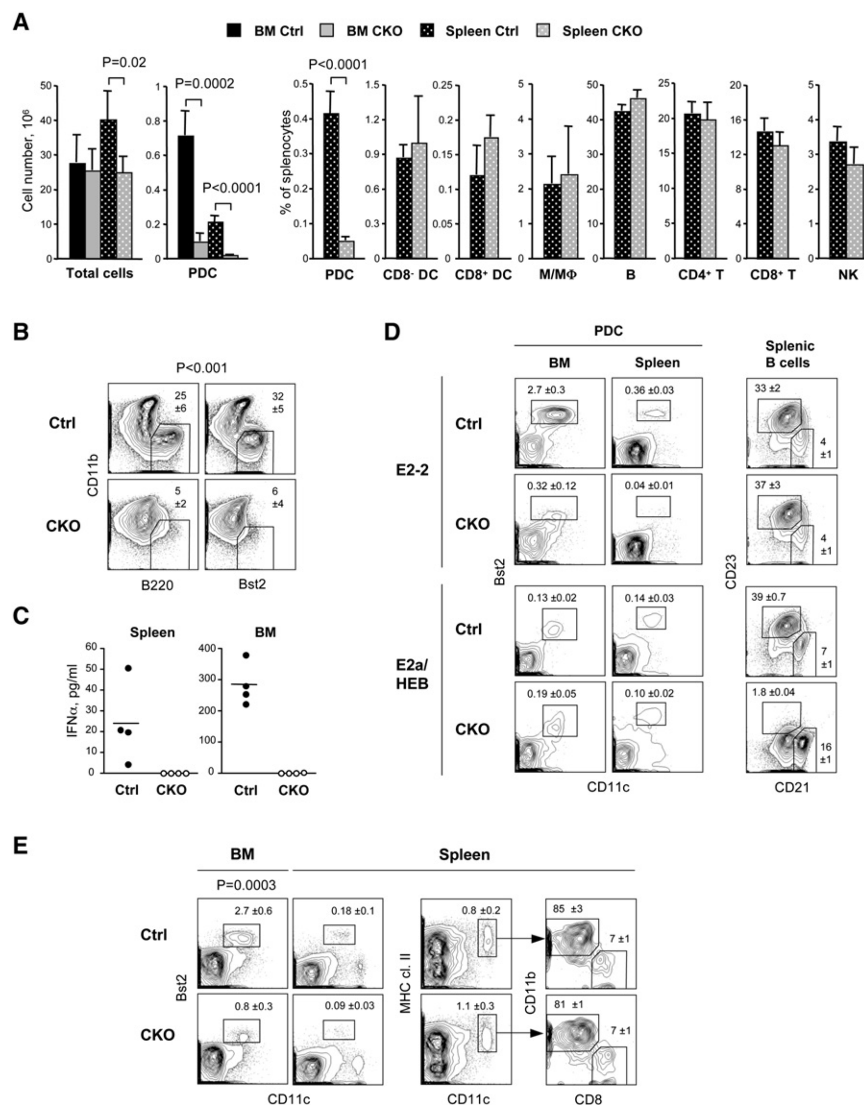

Figure 3. Conditional Inactivation of E2-2 Impairs PDC Development

(A) The analysis of *R26*-CreER$^+$ control (Ctrl, *E2-2*$^{wt/wt}$) and conditional knockout (CKO, *E2-2*$^{flox/flox}$) mice 10 days after the induction of Cre recombination by tamoxifen. Shown are absolute numbers of total cells and of the PDCs in the spleen and BM and fractions of indicated cell types in the spleen (mean ± SD of four animals per genotype). Cells included PDCs (CD11c^{low} Bst2$^+$), B cells (CD11c$^-$ B220$^+$), monocytes and macrophages (M/MΦ, CD11c$^-$ CD11b$^+$ side scatter^{low}), cDCs (CD11c^{high} MHC cl. II$^+$, CD8$^-$, or CD8$^+$), T cells (TCRβ$^+$, CD4$^+$, or CD8$^+$) and NK cells (DX5$^+$).

(B) PDC development in Flt3L-supplemented BM cultures from induced control and CKO mice. Shown are representative staining profiles of CD11c$^+$ cells from day nine BM cultures; CD11b$^-$ B220$^+$ or Bst2$^+$ PDCs are indicated (mean percentage ± SD of cultures from four individual mice).

(C) CpG-induced secretion of IFNα by total splenocytes and BM cells from induced control and CKO mice. Cells were incubated with type A CpG for 48 hr, and IFNα concentration in the supernatant was determined by ELISA. Symbols represent cell cultures from individual mice.

(D) Tamoxifen-induced deletion of E proteins in adult animals. *R26*-CreER$^+$ mice conditional for E2-2 (*E2-2*$^{flox/flox}$) or E2a/HEB (*E2a*$^{flox/flox}$ *HEB*$^{flox/flox}$) were analyzed 10 days after the induction. Wild-type C57BL/6 mice were used as controls for E2a/HEB CKO. Shown are gated B220$^+$ BM or spleen cells stained for PDCs (CD11c^{low} Bst2$^+$) or gated B220$^+$ AA4.1$^-$ splenocytes stained for follicular (CD23^{high} CD21low) or marginal zone (CD23^{low} CD21^{high}) B cells. The fractions of cell populations among total BM or spleen cells are indicated (mean ± SD of two to three animals per genotype).

(E) The analysis of *CD11c*-Cre$^+$ control (*E2-2* $^{wt/wt}$) and CKO (*E2-2*$^{flox/flox}$) animals. Shown are representative staining profiles of total BM and spleen cells from control and CKO mice, with the percentage of PDC, cDC, and cDC subsets indicated (mean ± SD of five animals per genotype).

E2-2$^{+/-}$ reporter mice compared to those from *E2-2*$^{+/+}$ littermates, suggesting an impaired induction of *IFNβ* gene (Figure 4D). Furthermore, serum IFNα was undetectable in CpG-challenged *E2-2*$^{+/-}$ mice (Figure 4E), whereas cDC-mediated serum IFN response to double-stranded RNA polymer poly-I:C (Kumagai et al., 2007) was not impaired. Furthermore, *E2-2*$^{+/-}$ mice mounted normal T cell-dependent antibody responses, suggesting that E2-2 reduction does not impair B cell function (Figure 4F). Thus, *E2-2* heterozygosity results in the reduced number of functionally impaired PDCs, revealing a haploinsufficiency of E2-2 for PDC development.

E2-2 Controls PDC Development in Humans

To test whether E2-2 plays a similarly important role in the human PDC, we analyzed human patients with Pitt-Hopkins syndrome (PHS). PHS is a rare autosomal-dominant genetic disorder characterized by abnormal craniofacial and neural development, severe mental retardation, and motor dysfunction. Recent studies identified monoallelic loss-of-function mutations or deletions of *TCF4* (*E2-2*) and the resulting E2-2 haploinsufficiency as a cause of PHS (Amiel et al., 2007; Zweier et al., 2007). We analyzed PDC phenotype and function in the peripheral blood from three PHS patients and five healthy control subjects. All major immune cell types, including naive and isotype-switched B cells, were present in normal numbers in PHS patients; similarly, the levels of serum immunoglobulins were normal (data not shown). The PDCs were present in all patients (Figure 5A) and expressed the expected high levels of CD45RA and CD123 (IL-3R); however, the expression of specific PDC marker BDCA-2/CD303 was significantly reduced (Figures 5B and 5C). The expression of ILT7 was more variable in controls, but uniformly low in the patients. Importantly, in vitro IFNα secretion in response to CpG was strongly reduced in all PHS patients, suggesting a severe functional defect of the PDC (Figure 5D). In contrast, phytohemagglutinin- and IL-2-induced secretion of IFNγ closely correlated with the number of CD56$^+$ CD3$^+$ cells and was not impaired

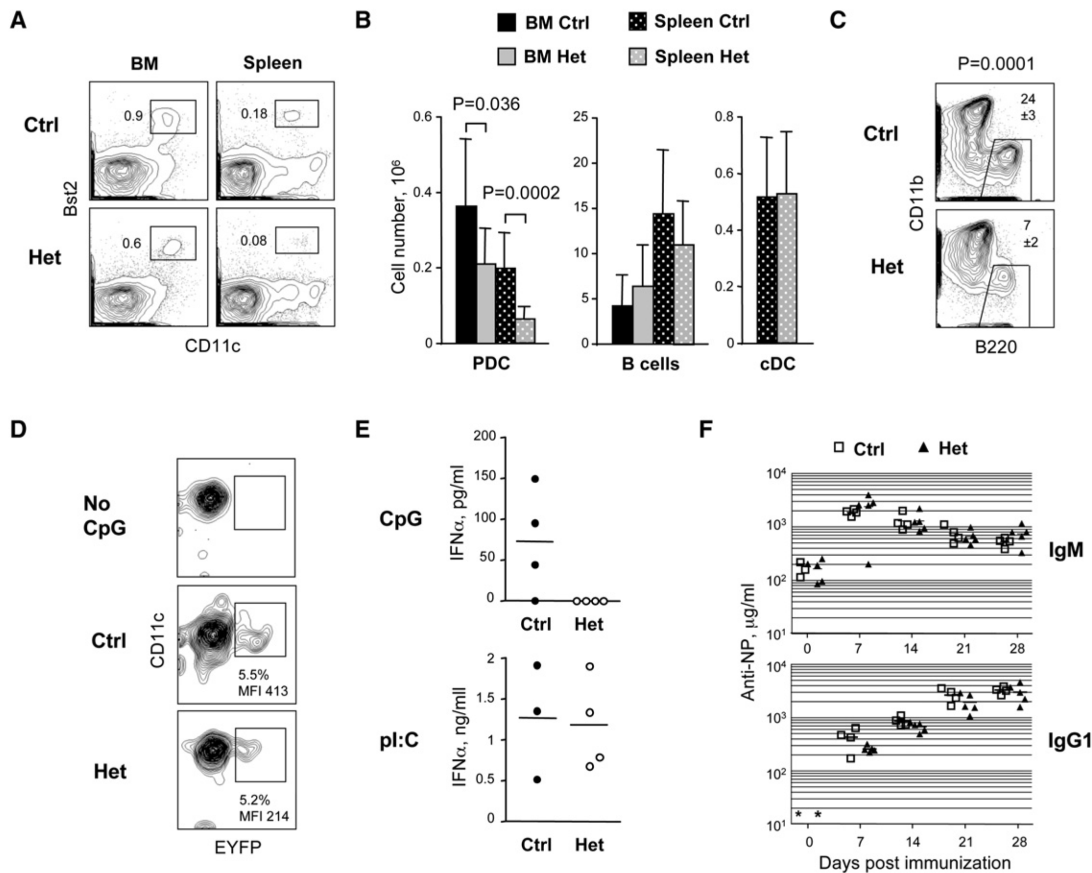

Figure 4. Impaired PDC Development and Function in E2-2 Heterozygous Mice

(A) Representative staining profiles of PDCs from the BM and spleen of $E2\text{-}2^{+/-}$ mice (Het) and wild-type control (Ctrl) littermates.

(B) Absolute numbers of PDCs ($CD11c^{low}$ $Bst2^{+}$) in $E2\text{-}2^{+/-}$ and control mice (mean ± SD of 8–11 mice per genotype). The numbers of splenic B cells ($B220^{+}$) and cDCs ($CD11c^{high}$ MHC cl. II^{+}) are also shown.

(C) PDC development in Flt3L-supplemented BM cultures from $E2\text{-}2^{+/-}$ and control mice. Shown are representative staining profiles of gated $CD11c^{+}$ cells from day 10 BM cultures; the PDC fraction is indicated (mean percentage ± SD of cultures from four individual mice).

(D) Induction of *IFNβ* gene expression in vivo. $E2\text{-}2^{+/-}$ mice or $E2\text{-}2^{+/+}$ littermate controls carrying one copy of *IFNβ*-EYFP knockin allele were injected with CpG/lipid complex, and EYFP expression was measured 6 hr later. Shown are EYFP fluorescence profiles of splenic PDCs ($B220^{+}$ $Bst2^{+}$) with the percentage and mean fluorescence intensity (MFI) of $EYFP^{+}$ cells indicated. Data are representative of four independent experiments (five to seven animals per genotype).

(E) Systemic IFN response after challenge with TLR ligands in vivo. $E2\text{-}2^{+/-}$ or control littermates were injected with TLR9 ligand CpG/lipid or with TLR3 ligand pI:C, and serum IFNα was measured 6 or 12 hr later, respectively. $E2\text{-}2^{+/-}$ mice similarly failed to mount IFN response to CpG after 12 hr (data not shown). Symbols represent IFNα concentrations in individual animals. The relatively low levels of CpG-induced IFNα are likely due to the early time point analyzed.

(F) T cell-dependent antibody responses in $E2\text{-}2^{+/-}$ and control mice. The mice were immunized with nitrophenyl (NP)-keyhole limpet hemocyanin (KLH) conjugate (50 μg in alum adjuvant), and serum anti-NP antibody titers were measured by ELISA at the indicated time points. Symbols represent individual control (open squares) or $E2\text{-}2^{+/-}$ (black triangles) immunized mice; asterisks indicate antibody levels below the detection limit. Similar results were obtained with IgG2a isotype (data not shown).

on a per-cell basis (data not shown). Thus, normal levels of E2-2 expression are essential for the development of functional PDCs in both mice and humans.

E2-2 Controls the Gene Expression Program of the PDC

To test whether E2-2 controls the specific gene expression program of PDCs, we performed genome-wide expression analysis of PDCs from $E2\text{-}2^{+/-}$ mice compared to wild-type controls (Document S3). Differentially expressed genes were analyzed against the database of gene expression in PDCs and other immune cell lineages (Robbins et al., 2008). Clustering based on the probe set downregulated in $E2\text{-}2^{+/-}$ cells separated PDCs away from all other cell types, suggesting that this set is highly enriched for PDC signature genes (Figure 6A). Indeed, several strongly downregulated genes (*Dntt*, *Mgl1*, *Ldhb*, *Ccr9*) are expressed predominantly in the PDC compared to other mature cell types (Figures 6B and 6C). The reduction of these genes in $E2\text{-}2^{+/-}$ PDCs was confirmed by qRT-PCR (Figure 6D), or by flow cytometry in the case of Ccr9 (Figure S7). These data demonstrate that E2-2 controls the expression of multiple PDC-enriched genes, including the genes common to PDCs and immature lymphocytes (such as *Dntt* and *Ccr9*).

Next, we focused on the genes involved in PDC development and/or function. The expression of Toll-like receptors *Tlr7* and

Figure 5. Impaired PDC Phenotype and Function in Human PHS Patients

Total peripheral blood cells from three PHS patients and five healthy controls were analyzed.

(A) Absolute number of PDCs (CD45RA$^+$ CD123$^+$ BDCA-2$^+$) in the peripheral blood as determined by flow cytometry. Symbols represent individual patients (PHS) and control subjects (Ctrl).

(B) Cell surface marker expression by PDCs. Total blood cells were simultaneously stained for the indicated PDC markers; shown are fluorescence histograms of the PDC population (gated as indicated) from a representative control and a PHS patient.

(C) The expression level of BDCA-2/CD303 on PDCs, shown as mean fluorescent intensity (MFI) of the BDCA-2$^+$ population.

(D) The secretion of IFNα in response to CpG in vitro. Total peripheral blood was incubated with type A CpG for 24 hr, and IFNα concentration in the supernatant was determined by ELISA.

Tlr9 and of the key IFN-inducing factor *Irf7* was reduced by ~30%, likely underlining the defective IFN response of *E2-2*$^{+/-}$ PDCs (Figure 6D). Furthermore, the levels of PDC-enriched transcription factors *SpiB* and *Irf8* was similarly reduced, suggesting that E2-2 may act upstream of these factors. To test this notion, we isolated BM culture-derived mature PDCs from naive *R26*-CreER$^+$ *E2-2*$^{flox/flox}$ CKO mice and cultured them in the presence of 4-hydroxytamoxifen (4-HT) to induce Cre-mediated E2-2 deletion. Compared to control *R26*-CreER$^+$ cells, CKO PDCs showed accelerated loss of *E2-2*, as well as of *SpiB* and *Irf8* transcripts (Figure 7A). The expression of *Irf7* was slightly accelerated compared to the nonspecific loss in control cells, whereas the levels of *Tlr7* and *Tlr9* were unchanged (data not shown). Thus, E2-2 is required for the expression of key transcription factors involved in PDC development (*SpiB*, *Irf8*) and function (*Irf7*).

Chromatin immunoprecipitation (ChIP) in the human PDC line CAL-1 revealed specific binding of E2-2 to the promoter fragments of human PDC markers *CLEC4C* (encoding BDCA-2/CD303), *LILRA4* (encoding ILT7), and *PTCRA*, consistent with the reduced expression of BDCA-2 (and possibly of ILT7) in the PDCs of PHS patients (Figure 7B). Moreover, E2-2 was also bound to the 5′ regions of *IRF8* and *IRF7*, suggesting that their E2-2-dependent expression in PDCs results from direct activation by E2-2. Indeed, the amplified promoter fragments of all these genes contain canonical E boxes (Figure S9). Because the expression pattern of both murine and human SpiB is very similar to that of E2-2, we sought a possible conserved E2-2-bound regulatory element in SPIB. We identified a highly conserved region in its first intron that contains two conserved E boxes (Figure 7C) and is bound by E2-2 in PDCs (Figure 7B). Thus, E2-2 directly controls the expression of PDC-enriched genes, including transcription factors critical for PDC development and function.

DISCUSSION

This work sought to define specific factors controlling the development of PDCs, the principal IFN-producing cell type that plays critical roles in both antiviral immunity and autoimmunity. We found that bHLH transcription factor E2-2 was preferentially expressed in the PDC and is selectively required for PDC development in a cell-intrinsic fashion. Although PDCs display some phenotypic and functional heterogeneity (O'Keeffe et al., 2002; Pelayo et al., 2005), all PDCs appeared to be E2-2 dependent. Consistent with the key role of PDCs in the IFN response to virus-associated nucleic acids, the deletion of E2-2 abolished IFN secretion in response to unmethylated DNA. Altogether, these data suggest that E2-2 is an essential and specific transcriptional regulator of PDC development and of PDC-mediated immune responses.

The identification of E2-2 as a regulator of the PDC helps elucidate several enigmatic aspects of PDC development. First, it explains the paradoxical expression of "early lymphoid" genes in mature PDCs, which occurs even in the PDCs experimentally derived from myeloid progenitors (Harman et al., 2006; Shigematsu et al., 2004). Here, we show that several genes common to PDCs and developing lymphocytes (murine *Dntt* and *Ccr9*, human *PTCRA*) are controlled or directly bound by E2-2; at the same time, these genes are major targets of E2a and/or HEB in lymphopoiesis (Dias et al., 2008; Reizis and Leder, 2001). Therefore, the shared expression profile of PDCs and of immature T and B lymphocytes does not necessarily reflect their common developmental origin but likely results from the activity of related E2-2 and E2a/HEB proteins in the respective lineages. Second, the immature cell population accumulating in *E2-2*$^{-/-}$ BM likely corresponds to an early stage of normal PDC development. This CD11c$^+$ population is more mature than the CD11c$^-$ pro-DCs (Naik et al., 2007; Onai et al., 2007) and has initiated development along the PDC lineage, as judged by the expression of PDC marker Ly-6C and by high recombination frequency in *CD11c*-Cre mice. Thus, E2-2 deficiency highlights a critical differentiation step in the BM between the early, immature PDC population and mature, functional PDCs.

Although all three E proteins are expressed in the PDC, we found that E2-2 is required for their development, whereas E2a and HEB are dispensable. Together with the EMSA supershift

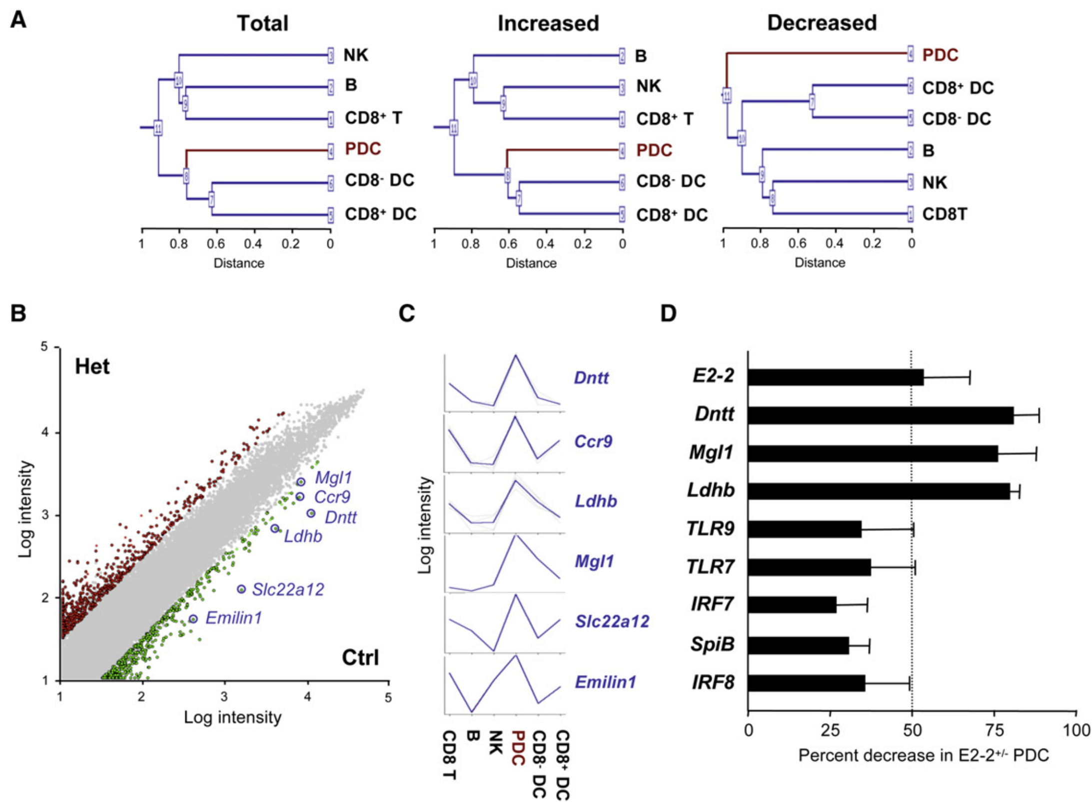

Figure 6. E2-2 Regulates the Expression of PDC-Enriched Genes

Sorted PDCs from $E2\text{-}2^{+/-}$ mice and wild-type littermate controls were analyzed by microarray expression profiling, and the results were compared to the expression database of normal immune cell populations (GEO dataset GSE9810).

(A) Clustering of cell populations by total probe set or by the probe sets increased or decreased in $E2\text{-}2^{+/-}$ PDCs.

(B) Pairwise comparison of $E2\text{-}2^{+/-}$ (Het) and control (Ctrl) PDC expression profiles. The scatter plot represents normalized log intensities of individual probes, with the probes increased or decreased >3-fold in $E2\text{-}2^{+/-}$ PDCs indicated in red and green, respectively. The probes prominently decreased in $E2\text{-}2^{+/-}$ PDCs are highlighted in blue.

(C) Expression profiles of the decreased probes from (B) in normal immune cell populations.

(D) Gene expression in sorted PDCs as determined by qRT-PCR. Data represent percent decrease of the indicated genes in $E2\text{-}2^{+/-}$ compared to control wild-type PDCs (mean ± SD of values from three to four independently sorted and analyzed PDC samples). The expected 50% decrease of E2-2 expression is indicated by the dotted line.

analysis, these data suggest that E2-2 acts primarily as a homodimer in PDCs. Whereas E2a protein levels are increased in activated B cells (Quong et al., 1999), the expression and activity of E2-2 were decreased upon PDC activation, reflecting the differentiation into another cell type, the cDC. Despite its expression in B cells, previous studies (Wikstrom et al., 2006; Zhuang et al., 1996) and our data suggest that E2-2 is largely dispensable for the development and maintenance of B cells. The reciprocal roles of E2-2 and E2a are likely explained by their relative abundance, which favors the formation of the respective homodimers in PDCs and in B cells. Because the expression level of each E protein gene appears to be perfectly tuned to its function, reduced gene dosage in heterozygotes results in haploinsufficiency. Thus, $E2a^{+/-}$ mice show impaired B cells development and selection and a specific reduction of splenic follicular B cells (Quong et al., 2004). Similarly, $E2\text{-}2^{+/-}$ mice contain lower numbers of PDCs; moreover, these PDCs show reduced expression of multiple PDC-enriched genes and are impaired in their IFN production capacity. The latter can be explained by the reduced expression of key IFN response components TLR7/9 and IRF7, likely in conjunction with other pathway components. Thus, the correct E2-2 expression level is required for the acquisition of mature PDC gene expression program and for the resulting IFN production capacity.

Recent studies revealed an evolutionarily conserved role of E2-2 in neural development. Thus, E2-2 deficiency abrogates the specification of pontine nucleus neurons in the mouse brain (Flora et al., 2007), whereas E2-2 haploinsufficiency leads to severe morphological and functional brain abnormalities in human PHS patients (Amiel et al., 2007; Zweier et al., 2007). Similarly, we found that E2-2 is preferentially expressed in both murine and human PDCs, in agreement with the recent microarray data (Robbins et al., 2008). Furthermore, PDCs from all three analyzed PHS patients showed aberrant surface phenotype and a profound defect of IFN secretion, similar to the results from $E2\text{-}2^{+/-}$ mice. Of note, at least two of the three analyzed

Figure 7. E2-2 Directly Activates PDC-Enriched Genes

(A) The expression of PDC-enriched transcription factors after E2-2 deletion in mature PDCs. Purified BM culture-derived PDCs from naive *R26*-CreER^{+} control (*E2-2*$^{wt/wt}$) or CKO (*E2-2*$^{flox/flox}$) mice were incubated with tamoxifen in vitro, and the expression of indicated transcripts was monitored by qRT-PCR. No differences in cell viability have been observed in the cultures (data not shown). The levels of all genes at day 1 were similar to those on day 0 and were taken as 100%. Data represent mean normalized values ± SD of triplicate PCR reactions.

(B) The binding of E2-2 to the regulatory regions of PDC-expressed genes. Chromatin immunoprecipitation was performed in human PDC line CAL-1 with anti-E2-2 or control antibodies, and the isolated chromatin was analyzed by qPCR. The data represent normalized fold enrichment of the indicated genes in anti-E2-2 compared to control antibody ChIP (mean ± SD of triplicate reactions). An irrelevant gene (*PROM1*) and an irrelevant internal fragment of *CLEC4C* gene (*CLEC4C**) were used as negative controls.

(C) The map of the human *SPIB* 5′ region and first intron, with the percent identity plot comparison to other genomes generated by ECR software (http://ecrbrowser.dcode.org). The conserved region in the first intron amplified by ChIP is highlighted with a red oval, and partial alignment of the human (top) and mouse (bottom) sequences is shown to the right. Conserved E boxes are indicated in red.

PHS patients have a history of recurrent respiratory tract infections, consistent with the defective IFN response of their PDCs. Although the rarity of the disease and ethical considerations precluded a broader analysis of PHS patients, these data support an evolutionarily conserved role of E2-2 in human PDC development.

How does E2-2 control PDC differentiation? The most obvious possibility is a direct induction of gene sets that would be critical for PDC commitment and/or lineage identity. Indeed, we found that several PDC-enriched genes (including bona fide specific markers such as BDCA-2) were reduced in human and/or murine *E2-2*$^{+/-}$ cells; moreover, E2-2 showed direct binding to the promoters of these genes. In addition, E2-2 controls the expression levels and directly binds to the regulatory regions of PDC-enriched transcription factors implicated in PDC development (*SpiB*, *IRF8*) and function (*IRF7*). Although neither gene is expressed or required exclusively in the PDC, the E2-2-initiated transcriptional network may control PDC-specific gene expression through the combined action of all these factors. Indeed, homologous transcription factors including E protein E2a, SpiB-related Ets protein PU.1, and IRF family proteins Irf4 and Irf8 comprise a critical part of B

lymphocyte regulatory network (Nutt and Kee, 2007; Singh et al., 2005). SpiB appears to be a prominent direct target of E2-2 because its expression is very similar to that of E2-2 and is rapidly decreased after E2-2 deletion; moreover, the identified region of E2-2 binding in the *SpiB* first intron is highly conserved. Thus, E2-2 serves as an upstream component of the complex genetic network that mediates PDC lineage commitment and differentiation.

E protein genes E2a and HEB are collectively required for every aspect of adaptive immune system development (Lazorchak et al., 2005; Murre, 2005). Conversely, the inhibition of E protein activity by Id2 protein is required for the development of several innate immunity cell types, such as natural killer (NK) cells and several cDC subsets (Hacker et al., 2003). We found that a key innate IFN-producing cell type lacks Id2 expression but instead is absolutely dependent on the third E protein, E2-2. These results not only confirm the critical function of all three E proteins in hematopoiesis but also extend it to the development of innate immune system. Notably, three E protein genes (as opposed to a single gene in invertebrates and chordates) are present in the genomes of jawed fish, which possess a V(D)J recombination-based lymphoid system as well as IFN response-related genes encoding IFN, IFN receptor, and IRF7. Thus, the diversification of E protein family appears to mirror the development of distinct lymphoid and IFN-producing lineages and is likely to play a role in these evolutionary steps.

In summary, our data identify E2-2 as an essential and specific transcriptional regulator of PDC development and of the PDC-mediated IFN response. These results open new possibilities for genetic and functional analysis of PDC lineage, including E2-2 deletion as a tool for specific PDC ablation, and genome-wide characterization of functional E2-2 target genes in PDCs. The observed role of E2-2 in the human PDC confirms the relevance of such studies for the human immune system. Because PDC hyperactivation contributes to autoimmune diseases (Banchereau and Pascual, 2006), E2-2-dependent molecular pathways may provide attractive targets for future drug development.

EXPERIMENTAL PROCEDURES

Animals

The *E2-2*$^{+/-}$ mice ([Zhuang et al., 1996], kindly provided by H. Zoghbi, Baylor College of Medicine) were backcrossed onto 129/SvEv background. For hematopoietic reconstitution, 14.5 days postcoitum (d.p.c.) embryos from heterozygous intercrosses were genotyped by PCR, and fetal liver cells from control (*E2-2*$^{+/+}$) and knockout (*E2-2*$^{-/-}$) embryos were pooled and injected into lethally irradiated (1000 Rad) B6.SJL mice (Taconic). The *IFN*β-EYFP strain carries EYFP reporter gene knocked into *IFN*β locus (S.S. and R.L., unpublished data). The *E2-2*^{flox} mice (Bergqvist et al., 2000) were crossed to *Rosa26*-CreER (*R26*-CreER, generated and kindly provided by T. Ludwig, Columbia University) and *CD11c*-Cre (Caton et al., 2007) strains. The *E2a*^{flox}/*HEB*^{flox} mice (Jones and Zhuang, 2007) were crossed to *R26*-CreER mice. All CKO mice were on C57BL/6 background. For the induction of Cre recombination in *R26*-CreER^{+} mice, tamoxifen was dissolved in oil and administered orally for three consecutive days (5 mg/day, E2-2 CKO) or injected intraperitoneally three times every other day (1 mg/day, E2a/HEB CKO), and the mice were analyzed 10 days after the first administration. All animal studies were performed according to the investigator's protocol approved by the Institutional Animal Care and Use Committee of Columbia University.

Human Subjects

Three PHS patients (3, 10, and 17 years of age) with confirmed monoallelic *E2-2/TCF4* mutations and five healthy adult controls were analyzed, as described in the Supplemental Experimental Procedures. The study was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen-Nuremberg, and informed consent was obtained in all cases.

Cell Analysis and Culture

Single-cell suspensions were stained with fluorochrome-conjugated antibodies to the indicated surface molecules (eBiosciences or BD PharMingen). The samples were acquired on LSR II flow cytometer or sorted on FACSAria flow sorter (BD Immunocytometry Systems) and analyzed with FlowJo software (Treestar). Magnetic enrichment for CD11b^{-} or B220^{+} PDCs was performed with MACS columns and microbeads (Miltenyi Biotech).

For PDC development in vitro, total BM cells (2×10^6/ml) were cultured for 8–10 days in complete Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum (FCS) with 100 ng/ml recombinant human Flt3L (Peprotech). For the induction of differentiation, PDCs from 8 day Flt3L cultures of wild-type BM were MACS purified and incubated for 48 hr with 1 μM type B CpG oligonucleotide (ODN 1826, Invivogen). For the induction of E2-2 deletion, PDCs from 8 day Flt3L cultures of *R26*-CreER^{+} control (*E2-2*$^{wt/wt}$) or CKO (*E2-2*$^{flox/flox}$) BM were MACS purified and incubated with Flt3L and 100 nM 4-hydroxytamoxifen (Sigma) for 1–4 days. Cell lines CAL-1 (Maeda et al., 2005) and MOLT-4 (ATCC #CRL-1582) were grown in complete RPMI 1640 with 10% FCS.

IFN Assay

Total BM or spleen suspensions (10^6/ml) were cultured in DMEM 10% FCS in the presence of 1 μM type A CpG (ODN 2216, Invivogen) for 48 hr. For the measurement of IFN induction in vivo, mice were injected intravenously with 5 μg ODN 2216 complexed with DOTAP (Roche; 30 μl DOTAP/150 μl total volume) or with 0.3 mg pI:C (GE Healthcare) and analyzed 6 hr or 12 hr later, respectively. IFNα concentration was measured by ELISA with anti-murine IFNα antibodies and recombinant standard (PBL Interferon Source).

Expression Analysis

Total RNA from purified human peripheral blood cell types (Allcells) or sorted murine cells was reverse transcribed and assayed by SYBR Green-based real-time PCR with MX3000P instrument (Stratagene). The expression of all genes was normalized to that of *β-actin* and expressed relative to the indicated reference sample via the $\Delta\Delta C_T$ method. All primers were validated for linear amplification (sequences available upon request). For microarray analysis and validation, PDCs (CD11c^{+} B220^{+} Bst2^{+}) were flow sorted from pooled spleens of *E2-2*$^{+/-}$ or wild-type littermates in multiple independent experiments. Microarray analysis was performed as described in the Supplemental Experimental Procedures.

Western Blotting, EMSA, and Chromatin Immunoprecipitation

Cell lysates of CAL-1 and MOLT-4 cell lines were probed with antibodies against human E2-2 ([Bain et al., 1993], monoclonal antibody [mAb] clone G108-391.2, kindly provided by C. Murre), E2a (mAb clone G98-271 reactive to E12/E47, BD PharMingen), and HEB (A-20, Santa Cruz Biotechnology). Electrophoretic mobility shift assay (EMSA) supershift was performed with the same antibodies as described in the Supplemental Experimental Procedures. For ChIP, CAL-1 cells were crosslinked with formaldehyde, sonicated, and subjected to immunoprecipitation with anti-E2-2 mAb or mouse IgG control. After crosslink reversal, the isolated chromatin was analyzed by qPCR as above. The level of all genes was normalized to 5% total chromatin input of anti-E2-2 or control IgG sample and expressed as fold difference between the two samples.

Statistical Analysis

Statistical significance was estimated with an unpaired, two-tailed Student's t test.

ACCESSION NUMBERS

The microarray data reported in this paper have been deposited in the GEO database with the accession numbers GSE12505 and GSE12507.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, nine figures, and two data files and can be found with this article online at http://www.cell.com/cgi/content/full/135/1/37/DC1/.

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