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Homeodomain only protein is required for the function of induced regulatory T cells in dendritic cell-mediated peripheral T cell unresponsiveness

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Abstract

Induced T regulatory (iT_{reg}) cells can be generated by peripheral dendritic cells (DCs) that mediate T cell-unresponsiveness to re-challenge with antigen. The molecular factors required for the function of such iT_{reg} cells remain unknown. We report a critical role for the transcription co-factor Homeodomain only protein (Hop, also know as Hopx) in iT_{regs} cells to mediate T cell unresponsiveness *in vivo*. Hopx-sufficient iT_{reg} cells down-regulate the expression of the AP-1 complex and suppress other T cells. In the absence of Hopx, iT_{reg} cells express high levels of the AP-1 complex, proliferate and fail to mediate T cell-unresponsiveness to re-challenge with antigen. Thus, Hopx is required for the function of T_{reg} cells induced by DCs and the promotion of DC-mediated T cell unresponsiveness *in vivo*.

The monumental task of distinguishing between potentially harmful entities bearing countless antigenic determinants and multiple self-proteins is a complex process referred to as immune tolerance that begins in the thymus and continues in the peripheral lymphoid system 1–6. In the thymus some developing T cells are deleted or become natural regulatory cells (nT_{reg} cells) that modulate immune responses in the periphery 1, 3–6. The active induction of tolerance can also continue extrathymically in the steady state when dendritic cells (DCs) constantly sample their cellular environment to process antigens for subsequent presentation to T cells and induction of immune tolerance 2. DC-induced tolerance to peripheral antigens is thought to be particularly important for limiting deleterious immune

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D.H. designed and performed the experiments, interpreted data and wrote the manuscript. Y.Y.W. produced FIR mice. E.E.E. contributed to the interpretation of data. R.A.F. oversaw the experimental design, interpreted data and wrote the manuscript.

responses to antigens unavailable during T cell development in the thymus though it could also limit beneficial anti-cancer immunity 1–4.

Induction of antigen-specific regulatory T cells is an important mechanism of peripheral tolerance induced by DCs under tolerogenic steady state conditions 7. In the mouse, such induced regulatory T (iT_{reg}) cells resemble thymic nT_{reg} cells in many respects and share an expression of some of the T_{reg} cell-specific genes including the transcription factor Foxp3 and the high affinity IL-2 receptor (CD25) 7. While the functions of thymus-derived nT_{reg} cells have been the focus of intense research over the last years, the specific mechanisms of induction and suppression operating in extrathymically iT_{reg} cells have only recently begun to be investigated 7–16. *In vivo*, the presentation of antigen by steady-state DCs leads to a burst of proliferation among the responding T cells but iT_{reg} cells arise from the few cells that proliferate only minimally during this initial antigenic stimulation 7. Such a low level of the proliferation that leads to induction of iT_{reg} cells by DCs is consistent with the classic anergic phenotype of T_{reg} cells 17–20. Although T_{reg} cells can proliferate and expand *in vivo*, the anergic phenotype of many T_{reg} cells has been intimately linked with their regulatory function to suppress T cell responses particularly those induced under strongly immunogenic conditions *in vivo* 17, 21, 22.

Anergy of T cells has been linked to disruption of specific signal transduction and cell cycle-controlling pathways and one of the best-known mechanisms is negative regulation of the function of the AP-1 complex 23–25. The AP-1 complex comprised of Fos and Jun transcription factors regulates expression of multiple genes that control cell proliferation and growth 26. The inhibition of the Fos or Jun transcriptional activity in anergic T cells results in the loss of their proliferation response to antigenic stimulation. In thymic nT_{reg} cells, inhibition of Fos or Jun transcription function by Foxp3 has been linked with their diminished proliferation upon T cell receptor (TCR) engagement but the specific mechanisms of anergy in iT_{reg} cells remain unknown 20, 27–29. In addition to its well-established post-translational regulation, the AP-1 complex has been recently found to be subject to transcriptional control by Homeodomain Only Protein (Hop also known as Hopx) that inhibits AP-1 expression by interfering with initiation of Fos transcription by Serum Response Factor (SRF) 30–33. However, the role of Hopx and its control of AP-1 in immune cells has remained unknown. Here we show that Hopx is critically required for peripheral T cell tolerance mediated by DCs which regulate T cell unresponsiveness during an immunogenic challenge with antigen. In our work, we report that the maintenance of anergic and regulatory phenotypes by DC-induced T_{reg} cell *in vivo* requires Hopx-dependent down-regulation of expression of Fos and Jun during a response to a secondary challenge with antigen. Thus, the transcription co-factor Hopx is required to mediate T cell unresponsiveness induced by steady-state DCs *in vivo* by playing a critical role for the function of iT_{reg} cells.

Results

Hopx-expressing T_{reg} cells mediate T cell unresponsiveness

In the steady-state DCs targeted with antigens *in vivo* induce immune tolerance, characterized by unresponsiveness of T cells to secondary re-challenge with cognate antigen

34, 35. This was established using targeted delivery of antigens such as ovalbumin (OVA) to DCs *in vivo* using an engineered hybrid antibody to DC-specific CD205 (DEC205) defined here as αDEC-OVA. αDEC-OVA carries peptide antigens as a fusion protein linked to the C terminus of its heavy chains for their subsequent presentation by DCs to antigen-specific T cells under steady state conditions 34. To characterize the molecular mechanisms which promote DC-mediated T cell tolerance, we used the same strategy to deliver antigens to DCs for presentation to T cells under steady-state conditions. We performed gene-array experiments using DC-tolerized antigen-specific total $CD4^+$ T cells in order to identify candidate genes responsible for DC-induced T cell unresponsiveness. Thus, we identified Homeodomain Only Protein (*Hop* or *Hopx*), a previously described co-factor involved in transcriptional regulation in non-lymphoid tissues 30, 31 to be induced in T cells tolerized by steady state DCs *in vivo* (data not shown). We verified the results of the gene-arrays by quantitative real-time PCR and found an approximately 5-fold increase of *Hopx* expression in OVA-specific CD4 T cells from OTII OVA-TCR transgenic (Tg) mice injected with αDEC-OVA (Fig. 1a). The targeted deletion of *Hopx* involving loss of all *Hopx* exons, results in a non-lethal defect in cardiac development but the immune system of the mutant mice develops without gross abnormalities 30, 31. We back-crossed *Hopx* -deficient ($Hopx^{-/-}$) mice that were generated on the 129 × B6 mixed background 30 for 11 generations onto the C57BL/6 background and then crossed them with anti-OVA TCR Tg (OTII) animals 36. To examine if expression of Hopx was required for DC-mediated T cell unresponsiveness, we used *Hopx* -sufficient and *Hopx* -deficient anti-OVA T cells isolated from OVA TCR Tg $Hopx^{+/-}$ and $Hopx^{-/-}$ OTII littermate mice injected with (αDEC-OVA). We labeled such T cells with 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) and measured their cell division in response to OVA in Complete Freund's Adjuvant (CFA) after adoptive transfer. Consistent with previously published results 35, the *Hopx*-sufficient T cells were unresponsive to further challenge with antigen *in vivo* and did not proliferate when re-challenged with OVA in CFA (Fig. 1b). In contrast, the transferred *Hopx* -deficient total $CD4^+$ T cells remained responsive to re-challenge with OVA in CFA (Fig. 1b). We conclude that Hopx is required for DC-mediated T cell unresponsiveness *in vivo*.

Previous research from other investigators showed that targeting DCs with antigens such as anti-DEC-antigen hybrid antibodies leads to the induction of iT regs 7. To examine whether Hopx is expressed in iT_{reg} cells induced by DCs *in vivo*, we isolated T cells from OVA TCR tg (OTII) mice treated with αDEC-OVA and determined *Hopx* expression by quantitative PCR in $CD4^+CD25^+$ iT_{reg} cells and $CD4^+CD25^-$ T cells of non-regulatory phenotype (Fig. 1c). Expression of *Hopx* in anti-OVA iT_{reg} cells was about 20 times higher than in anti-OVA $CD4^+CD25^-$ T cells isolated from the same αDEC-OVA-treated OTII mice (Fig. 1c). In contrast, the $CD25^+Foxp3^+$ nT_{reg} cells that develop in the thymus express *Hopx* at levels about 3 times lower than in iT_{reg} cells induced *in vivo* (Supplementary Fig. 1) We conclude that Hopx is preferentially expressed in iT_{reg} cells during the induction of DC-mediated T cell unresponsiveness.

Research from other laboratories 7, 37 has established that $CD4^+CD25^+$ T cells induced by steady-state DCs have a regulatory phenotype characterized by expression of Foxp3,

however the expression of Hopx by these cells remained unexplored. To analyze the function of such Hopx-expressing T_{reg} cells induced by DCs, we depleted OTII $CD4^+$ T cells isolated from αDEC-OVA-treated OTII mice of $CD4^+CD25^+$ double positive iT_{reg} cells, and labeled the remaining ($CD25^-$) cells with CFSE to track their cell division. $CD25^-$ T cells were then transferred into CD45.1 recipient mice either alone or after reconstitution with the originally depleted iT_{reg} cells, to attain the original frequency (5%) of $CD4^+CD25^+$ T cells. The recipient mice were subsequently challenged with OVA in CFA. In the absence of iT_{reg} cells, the transferred anti-OVA $CD25^-$ T cells proliferated to re-challenge with OVA in CFA, but the presence of iT_{reg} cells restored T cell unresponsiveness (Fig. 1b and Fig. 1b). We conclude that iT_{reg} cells are required for DC-induced T cell unresponsiveness *in vivo*.

Unaltered development of *Hopx*-deficient T_{reg} cells

We found that both Hopx and the iT_{reg} cells that express Hopx are required for DC-mediated T cell unresponsiveness *in vivo* (Fig. 1). We therefore, reasoned that Hopx might play a role in either the homeostasis or suppressor function of iT_{reg} cells induced by DCs *in vivo*. To examine the role of Hopx in iT_{reg} cells, we first compared the numbers of OVA-specific iT_{reg} cells in the $Hopx^{-/-}$ and $Hopx^{+/-}$ mice treated with αDEC-OVA. We injected $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII littermate mice with αDEC-OVA as in Fig. 1 and performed FACS analysis of isolated T cells. We found similar numbers (5.1% versus 5%) of OVA-specific $CD4^+CD25^+$ double-positive iT_{reg} cells in αDEC-OVA-treated $Hopx^{-/-}$ and $Hopx^{+/-}$ littermates (Fig. 2a). Thus, Hopx does not determine the number of iT_{reg} cells induced by DCs.

Foxp3 is a signature transcriptional regulator required for the function of T_{reg} cells of thymic origin (i.e. nT_{reg} cells) which is also expressed in iT_{reg} cells 7, 38, 39. To determine if the expression of Foxp3 in T_{reg} cells induced by DCs was affected by absence of *Hopx*, we performed Foxp3 intracellular staining in OVA-specific $CD4^+CD25^+$ double-positive iT_{reg} cells from αDEC-OVA-treated $Hopx^{-/-}$ and $Hopx^{+/-}$ littermates. Both $Hopx^{-/-}$ and $Hopx^{+/-}$ iT_{reg} cells expressed a similar level of Foxp3 and were present at a similar percentage (87% versus 88%) (Fig 2b).

In T_{reg} cells of thymic origin, Foxp3 was proposed to amplify and maintain the genetic signature that is induced by various developmental and environmental cues and is required for the anergic and suppressive phenotype in T_{reg} cells 20, 27, 28. To examine if Hopx affects the gene expression pattern that is characteristic for Foxp3 in T_{reg} cells, we crossed $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII mice with Foxp3-IRES-red fluorescent protein (RFP) reporter (FIR) mice 40 and determined the expression of several known genes specific for $Foxp3^+$ T_{reg} cells in $Hopx^{-/-}$ and $Hopx^{+/-}$ RFP^+ ($Foxp3^+$) iT_{reg} cells from αDEC-OVA-treated OTII-FIR mice. We found similar levels of expression of the $Foxp3^+$ T_{reg} cell-associated molecules *Nrp1*, *Icos*, *Ctla4* and *Gitr* in $Hopx^{-/-}$ and $Hopx^{+/-}$ iT_{reg} cells (Fig. 2c). We conclude that absence of *Hopx* does not alter the numbers of iT_{reg} cells induced by DCs, nor does it obviously disrupt the $Foxp3^+$-specific gene expression pattern in these cells.

Hopx-deficient T_{reg} cells lose suppressor function

To investigate directly the role of Hopx in the suppressor function of iT_{reg} cells induced by DCs *in vivo*, we compared the suppressor function of $Hopx^{-/-}$ and $Hopx^{+/-}$ iT_{reg} cells from αDEC-OVA-treated $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII littermate mice. We first CFSE-labeled *Hopx*-sufficient OVA-specific $CD25^-$ T cells and reconstituted them with *Hopx*-sufficient or *Hopx*-deficient OVA-specific $CD25^+$ iT_{reg} cells to obtain the original frequency (5%) of $CD4^+CD25^+$ T cells which is shown in Fig. 2b or with saline control (PBS) only. We adoptively transferred these different combinations of cells into CD45.1 recipient mice that we then immunized with OVA in CFA. To measure the response of $CD25^-$ T cells to re-challenge with OVA, we followed their proliferation *in vivo* by tracking CFSE dilution as in Fig. 1 (Fig. 2d). Similar to the results from Fig. 1, we found that in the presence of *Hopx*-sufficient iT_{reg} cells, $CD25^-$ T cells from αDEC-OVA-treated mice were unresponsive to re-challenge with OVA in CFA *in vivo* but proliferated in the absence of the iT_{reg} cells (PBS only) (Fig. 2d and compare Fig. 1c). In contrast, $CD25^-$ T cells responded to re-challenge with antigen when reconstituted with *Hopx*-deficient iT_{reg} cells (Fig. 2d). In contrast to $CD25^-$ T cells, the vast majority of $CD25^+$ iT_{reg} cells expressed Foxp3 (Fig 2b and Supplementary Fig. 2). In a separate experiment (Supplementary Fig. 3) we used pure $Foxp3^+$ iT_{reg} cells from αDEC-OVA treated $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII-FIR littermate mice and determined that only *Hopx*- sufficient but not *Hopx*-deficient $Foxp3^+$ iT_{reg} cells could suppress proliferation to re-challenge *in vivo* (Supplementary Fig. 3). Our results are also consistent with the lack of DC-induced T cell unresponsiveness observed in transferred total *Hopx*-deficient $CD4^+$ T cells after re-challenge with OVA in CFA (Fig. 1b). We conclude that expression of Hopx is required for the suppressor function of T_{reg} cells induced by DCs. In contrast, Hopx is not required for the function of nT_{reg} cells because *Hopx*-deficient $CD25^+Foxp3^+$ nT_{reg} cells remain functional (Supplementary Fig. 4).

To exclude a direct function of Hopx in non-regulatory ($CD4^+CD25^-$) T cells during DC-mediated T cell unresponsiveness, we examined whether *Hopx*-deficient $CD25^-$ T cells were susceptible to suppression by *Hopx*-sufficient iT_{reg} cells. We obtained iT_{reg} cells and $CD25^-$ T cells as in Fig. 2d and transferred the CFSE-labeled *Hopx*-deficient $CD25^-$ T cells that were reconstituted with either *Hopx*-sufficient or *Hopx*-deficient $CD25^+$ iT_{reg} cells or with PBS only. We then measured cell division of *Hopx*-deficient $CD25^-$ T cells in response to immunization with OVA in CFA. *Hopx*-deficient OVA-specific $CD25^-$ T cells in the presence of *Hopx*-sufficient iT_{reg} cells became unresponsive to further challenge with antigen *in vivo* and did not proliferate when re-challenged with OVA in CFA (Fig. 2e). By contrast, in the absence of iT_{reg} cells or in the presence of *Hopx*-deficient iT_{reg} cells, the *Hopx*-deficient OVA-specific $CD25^-$ T cells remained responsive and proliferated to re-challenge with antigen (Fig. 2e). We conclude that Hopx is not required for $CD25^-$ non-regulatory T cells to be susceptible to suppression by T_{reg} cells induced by DCs. We further conclude that a Hopx-dependent suppressor function of iT_{reg} cells is both necessary and sufficient for DC-mediated T cell unresponsiveness to antigenic re-challenge *in vivo*.

Hopx-dependent gene expression in iT_{reg} cells

Hopx is a recently identified, small homeodomain protein, evolutionarily highly conserved between rodents and humans, that has an indirect transcription-regulatory activity 30, 31.

Hopx interferes with transcription of some immediate early genes such as *Fos* and it can influence expression of other diverse genes in various non-lymphoid tissues and organs including heart, skeletal muscles, lungs and nervous system 30–33. We found that the absence of *Hopx* in the steady-state does not affect the generation of T_{reg} cells by DCs or their expression of $Foxp3^+$-specific genes (Figs. 2a–c). However, during re-challenge with antigen *in vivo* the suppressor function of such T_{reg} cells is compromised in the absence of *Hopx* (Fig. 2d and e). To define the role of Hopx in regulating gene expression in iT_{reg} cells during the antigenic re-challenge, we compared total gene expression in the presence or absence of Hopx in iT_{reg} cells in response to immunization. We immunized with OVA in CFA the $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII-FIR littermate mice that we first pre-treated with αDEC-OVA, and then compared global gene expression in isolated $Hopx^{-/-}$ and $Hopx^{+/-}$ anti-OVA $CD25^+RFP^+$ ($Foxp3^+$) double-positive iT_{reg} cells. We identified several proliferation-related genes that were altered in the absence of *Hopx* (Fig. 3). This result is in agreement with the established role of Hopx as a negative transcriptional regulator of the expression of the AP-1 complex and/or *Fos* 30–33. We conclude that Hopx alters expression of proliferation-promoting genes in iT_{reg} cells induced by DCs upon re-challenge with antigen *in vivo*.

In contrast, we found multiple genes that are known targets of other transcription factors such as Foxp3 or which are associated with separate T cell lineages including nT_{reg}, T_R1, T_H1, T_H2 and T_H17 cells, to be expressed at similar levels in the presence and absence of Hopx in iT_{reg} cells after the re-challenge with antigen (Fig. 3, Table 1 and Supplementary Figs. 5–7).

Hopx controls anergy of DC-induced T_{reg} cells

Unresponsiveness to antigen (anergy) is an integral part of the function of some T_{reg} cells but the molecular mechanisms that induce and maintain anergy in such cells remain unclear 17–20, 41. Since our results suggested a role for Hopx in T_{reg} cells induced by DCs in controlling expression of *Fos*, *Jun* and cyclin genes all of which are known to be involved in mediating T cell proliferation and anergy 23–25, we examined expression of these genes in such iT_{reg} cells both in the steady-state and after re-challenge with antigen. Expression of Fos and Jun was similar in *Hopx*-sufficient and *Hopx*-deficient anti-OVA $CD25^+RFP^+$ ($Foxp3^+$) double-positive iT_{reg} cells that were isolated from $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII-FIR littermate mice treated with αDEC-OVA. We then compared gene expression of Fos and Jun in $Hopx^{-/-}$ and $Hopx^{+/-}$ anti-OVA $CD25^+RFP^+$ ($Foxp3^+$) double-positive iT_{reg} cells that were isolated from $Hopx^{-/-}$ and $Hopx^{+/-}$ OTII-FIR littermate mice first pre-treated with αDEC-OVA and then immunized with OVA in CFA 12 or 24 hours prior to isolation of the cells (Fig. 4a and b). Consistent with the known down-regulation of AP-1 (Fos and Jun) complexes that occurs during the induction of anergy 23–25, we observed a 3–4 fold decrease in expression of Fos and Jun in *Hopx*-sufficient iT_{reg} cells 24h after re-challenge with OVA in CFA as compared to the level of expression in the steady state (Fig. 4a and b). In contrast, the expression levels of *Fos* and *Jun* in *Hopx*-deficient iT_{reg} cells remained at high levels after the re-challenge with antigen (Fig. 4a and b). We conclude that Hopx is required to down-regulate expression of proliferation-promoting Fos and Jun in iT_{reg} cells *in*

vivo upon antigenic re-challenge and this result suggests a role for Hopx in regulating anergy of such cells.

Cyclin D1 is one of the major Fos targets during initiation of cell proliferation 26. In the steady-state, expression of Cyclin D1 was about 35 % lower in *Hopx*-deficient iT_{reg} cells induced by DCs (Fig. 4c). We compared cyclin D1 gene expression in *Hopx*-sufficient and *Hopx*-deficient iT_{reg} cells in response to re-challenge with OVA in CFA (Fig. 4c). At 12 h following re-challenge, Cyclin D1 expression was decreased by ~80% in *Hopx*-sufficient iT_{reg} cells but its expression remained at high levels in the absence of Hopx. We conclude that down-regulation of cyclin D1 gene expression occurs concomitant with decreased expression of *Fos* and *Jun* in *Hopx*-sufficient iT_{reg} cells but expression of cyclin D1 remains high in the absence of *Hopx*.

The Hopx-dependent down-regulation of proliferation-promoting genes after re-challenge with antigen in T_{reg} cells induced by DCs suggests that Hopx is required to maintain anergy of these cells during the acute phase of an antigenic re-challenge response. To examine directly whether Hopx controls proliferation of iT_{reg} cells in response to re-challenge with OVA in CFA, we adoptively transferred CFSE-labeled *Hopx*-sufficient and *Hopx*-deficient anti-OVA iT_{reg} cells from αDEC-OVA-treated OTII mice into CD45.1 recipient mice that we then immunized with OVA in CFA; we followed proliferation of these transferred iT_{reg} cells *in vivo* by tracking dilution of CFSE (Fig. 4d). Consistent with the anergic phenotype of T_{reg} cells, we found that the majority of transferred *Hopx*-sufficient iT_{reg} cells induced by DCs did not proliferate (63% of CFSE-high [undivided] cells remaining 3d after the re-challenge with antigen) (Fig.4 d). In contrast, *Hopx*-deficient iT_{reg} cells induced by DCs responded by proliferating to re-challenge with OVA in CFA (39% of CFSE-high [undivided] cells remaining 3d after the re-challenge with antigen) (Fig. 4d). Thus, consistent with its role in down-regulating proliferation-promoting genes (Fig. 4a–c), Hopx is required for the maintenance of the continuous state of anergy in iT_{reg} cells cells upon re-challenge with antigen.

Discussion

DCs were initially discovered to induce immune responses under inflammatory conditions and they have been fittingly called “nature’s adjuvants” 42–44. In the steady-state however, DCs are powerful antigen presenting cells that are constantly sampling their cellular environment to process antigens for subsequent presentation to T cells and induction of immune tolerance 2. Induction of antigen-specific iT_{reg} cells is an important mechanism of peripheral tolerance induced by DCs under tolerogenic steady-state conditions but the specific molecular factors required for the function of DC-induced T_{reg} cells have remained unknown 7–13. We have identified *Hopx* 30, 31 expression by iT_{reg} cells induced by DCs in the periphery to be indispensable for these lymphocytes to mediate DC-dependent T cell unresponsiveness. *Hopx*-deficient iT_{reg} cells fail to remain anergic and suppress T cell responses during immunogenic challenge with antigen.

In contrast, the $CD25^{+}Foxp3^{+}$ nT_{reg} cells that develop in the thymus express *Hopx* at levels about 3 times lower than in iT_{reg} cells induced *in vivo*. Moreover, Hopx is not required for

the function of nT_{reg} cells because *Hopx*-deficient $CD25^+Foxp3^+$ nT_{reg} cells remain functional Also, *Hopx* mutant mice appear to have normal tolerance to systemic antigens presented in the thymus 30, 31.

The seminal discovery of Foxp3 as a transcription factor specific for nT_{reg} cells has triggered great interest in these lymphocytes although the precise molecular mechanisms responsible for their anergic-suppressive phenotype still remains incompletely understood 20, 27, 28, 38, 39. The classic anergic phenotype is consistent with the minimal proliferation required for the initial generation of iT_{reg} cells by DCs *in vivo* 7. Although T_{reg} cells can proliferate and expand *in vivo* particularly under homeostatic conditions, anergy has been intimately linked with the regulatory function of many T_{reg} cells to suppress acute T cell responses induced under strongly immunogenic conditions 17, 21, 22. The precise molecular link between anergy in nT_{reg} cells and their various proposed mechanisms of direct suppression remains to be fully understood, but it is well established that Foxp3 is required for anergy 20, 27–29, 45. Absence of Foxp3 results in increased proliferation of nT_{reg} cells upon TCR engagement and Foxp3 has also been shown to inhibit proliferation of nT_{reg} cells by binding to Jun and diminishing its transcription function 20, 29.

We now find that DC-induced T_{reg} cells express *Hopx* which then maintains a state of non-proliferation in those cells upon re-challenge with antigen under immunogenic conditions. Hopx acts in the presence of Foxp3 but the specific function of Hopx appears to be distinct from the transcriptional role of Foxp3 because both the generation of $Foxp3^+$ T cells and expression of Foxp3-dependent genes in DC-induced T_{reg} cells are unaltered in the absence of *Hopx*. Foxp3 was proposed to amplify and maintain the genetic signature of nT_{reg} cells induced by various developmental and environmental cues, rather than act as a “master switch” that turns on an anergic-suppressor phenotype 20, 27, 28 and it is unknown at present if Hopx’s function could be affected by products of the Foxp3 transcription program.

We also examined the expression of several surface antigens and immunomodulatory cytokines that are known to participate in T cell regulation and suppression 13, 46, 47 and found no difference in their expression in absence of Hopx. In contrast, the previously unappreciated molecular function of Hopx in DC-induced T_{reg} cells is consistent with its established direct role as a negative transcriptional regulator of proliferation-promoting genes such as *Fos* whereby it prevents ternary complex factors such as SRF 48 from transcribing *Fos* and other immediate-early genes upon cellular activation 30, 31. This function of Hopx in iT_{reg} cells further provides a molecular explanation for the established mechanism of T cell anergy which is a down-regulation of the function of the AP-1 complex even though the role of Hopx in iT_{reg} cells generated by other approaches in the absence of DC remains unknown at this point 23–25. While the complete explanation of a deficient suppression by non-anergic T_{reg} cells will require considerable further study it is well established that Foxp3 regulates both anergy and suppression in nT_{regs} cells 17, 20, 29. Analogous to this established function of Foxp3 to mediate both anergy and suppression, Hopx is required to maintain suppressive function by anergic iT_{reg} cells during acute immunogenic responses. Following antigenic re-challenge, *Hopx*-deficient iT_{reg} cells cannot down-regulate expression of *Fos*, *Jun* and other proliferation-promoting genes and they both

lose their anergic phenotype and fail to inhibit proliferation of antigen-specific T cells. Thus, Hopx maintains the state of T cell unresponsiveness induced by steady-state DCs *in vivo*.

Methods

Gene arrays

Total gene expression in freshly isolated DC-tolerized and un-activated T cells was measured using Affymetrix Mouse Genome 430 2.0 Arrays. The final processing of RNA, hybridizations and primary data collection were performed at the Keck Affymetrix Facility of Yale University. The data obtained from all experimental groups were analyzed using the generic GCOS Affymetrix software, ArrayAssist software from Stratagene and GeneSpring. For the analysis we used gene expression profiles of the un-activated T cells from PBS injected mice as the baseline and in our initial analysis we focused primarily on genes involved in transcriptional regulation. To identify Hopx-dependent genes, we compared total gene expression in *Hopx*-deficient and *Hopx*-sufficient TCRα2^{+}CD4^{+}CD25^{+}RFP^{+} (Foxp3^{+}) DC-induced T_{reg} cells using the same methodology as above.

Mice

Hopx$^{-/-}$ mice 30 on a mixed 129/B6 background were backcrossed 11 generations with C57BL/6 mice before crossing them with OTII TCR Tg 36 and Foxp3-IRES-RFP (FIR) 40 animals. Experimental animals were produced by breeding the *Hopx*$^{+/-}$ with *Hopx*$^{+/-}$ or *Hopx*$^{-/-}$ mice. Age (7–10 weeks old) and sex-matched *Hopx*-deficient and sufficient littermates were used for the experiments. All mice were maintained in our facility under specific pathogen-free conditions and used in accordance with committee-approved protocols of the Yale University School of Medicine animal facility

Production of hybrid antibodies

DNA coding for the part of the OVA protein (320–347) containing the OTII peptide (323–339) with spacing residues on both sides was cloned into the C terminus of the heavy chains of a hybrid anti-DEC-205 34 using synthetic oligonucleotides:

Forward:

5’
CTAGCGACATGGCCAAGAAGGAGACAGTCTGGAGGCTCGAGGAGTTCGG
T

AGGTTCGAGAGCCTGAAGATATCTCAAGCT

GTCCATG

5’CAGCACATGCAGAAATCAATGAAGCAGGCAGAGAGGTGGTAGGGTCAG
CA

GAGGTCATGGAGGAGAGGTAATAGGC

Reverse:

5’GGCCGCCTATTACCTCTCCTCCATGACCTCTGCTGACCCTACCACCTCTC TG

CCTGCTTCATTGATTTCTGCATGTGCTG

CATGGACA

5’GCTTGAGATATCTTCAGGCTCTCGAACCTACCGAACTCCTCGAGCCTCC AG

ACTGTCTCCTTCTTGGCCATGTCG

Hybrid antibodies were expressed in 293T cells by transient transfection using a calcium phosphate method. Cells were grown in serum free DMEM supplemented with Nutridoma SP (Roche). Antibodies were purified on Protein G columns (Roche). The concentrations of antibodies were determined by ELISA using goat anti-mouse IgG1 and mouse IgG1 antibodies as a standard (Jackson Immunotech). Hybrid antibodies were injected in PBS intraperitoneally.

Flow cytometry and antibodies used for staining

Anti-CD4- (L3T4), anti-CD25 (PC61 and 7D4), anti-Valpha2 (B20.1), anti-Vbeta5.1, 5.2 (MR9-4), anti-CD45.2 (104), antu-Il-10 (JES5-16E3) were from BDPharmingen. Anti-Foxp3 (FJK16s), anti-CD73 (TY/11.8), anti-CD39 (24DMS1) were from eBioscience. Flow cytometry and cell sorting (including separation based on RFP expression) was performed on FACSCALIBUR and FACS Vantage (BD). For intracellular staining cells were fixed and permeabilized using Fixation-Permeabilization buffers from eBioscience and BD according to manufacturers’ manual. For cytokine stainings cells were additionally re-stimulated with PMA and Ionomycin.

Adoptive transfer

CD4, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from OTII transgenic mice were isolated by depletion of $CD8^+$, $B200^+$, $CD11c^+$, $CD11b^+$ and $NK1.1^+$ cells with magnetic microbeads (Miltenyi) and subsequent automated cell sorting FACS Vantage (BD). $CD4^+$ and $CD4^+$ CD25- or $CD4^+$ RFP- cells were labeled with 3 μM CFSE (Molecular Probes) in 5% FCS RPMI at 37°C for 25 min and washed 3× with PBS and 5×10^6 cells injected intravenously per mouse. In experiments shown in Figs. 1D, 2D and 2E, 5×10^6 $CD4^+CD25^-$ or $CD4^+$ RFP- cells were reconstituted with 0.3×10^6 $CD4^+CD25^+$ or $CD4^+RFP^+$ cells before they were injected intravenously per mouse. In experiments shown in Fig. 4D, 0.3×10^6 CFSE-labeled $CD4^+RFP^+$ cells were transferred.

Immunizations

Synthetic OVA peptide (323–339) (Yale Keck Protein Synthesis Facility) was injected in Complete Freund’s Adjuvant (Difco) subcutaneously.

Real-time RT- PCR analysis

RNA was isolated from the sorted peripheral CD4 T cells using TRIZOL Reagent (Invitrogen) and Qiagen mRNAEasy kit (Qiagen). Total RNA was reverse transcribed and

the cDNA was subsequently used for real-time PCR on an ABI Prism instrument using commercial primer-probe sets (Applied Biosystems): HOPX- Mm01217753_m1, ICOS-Mm00497600_m1, Nrp1- Mm00435379_m1, CTLA-4- Mm00486849_m1, GITR-Mm00437136_m1, Helios-Mm00496108_m1, Foxp3- Mm00475165_m1, Fos-Mm00487425_m1, Jun- Mm00495062_s1, Ccnd1- Mm00432359_m1, IL12a(p35)-Mm00434165_m1, Il-10-Mm00439616_m1, Tgfb1- Mm00441724_m1, Hypoxanthineguanine phosphoribosyltransferase (HPRT) was included as an internal control. The results of Q-PCR were standardized to the HPRT expression levels and analyzed by the dd CT method.

Statistical analysis

P values were calculated using Student's t-test, error bars in graphs represent SD

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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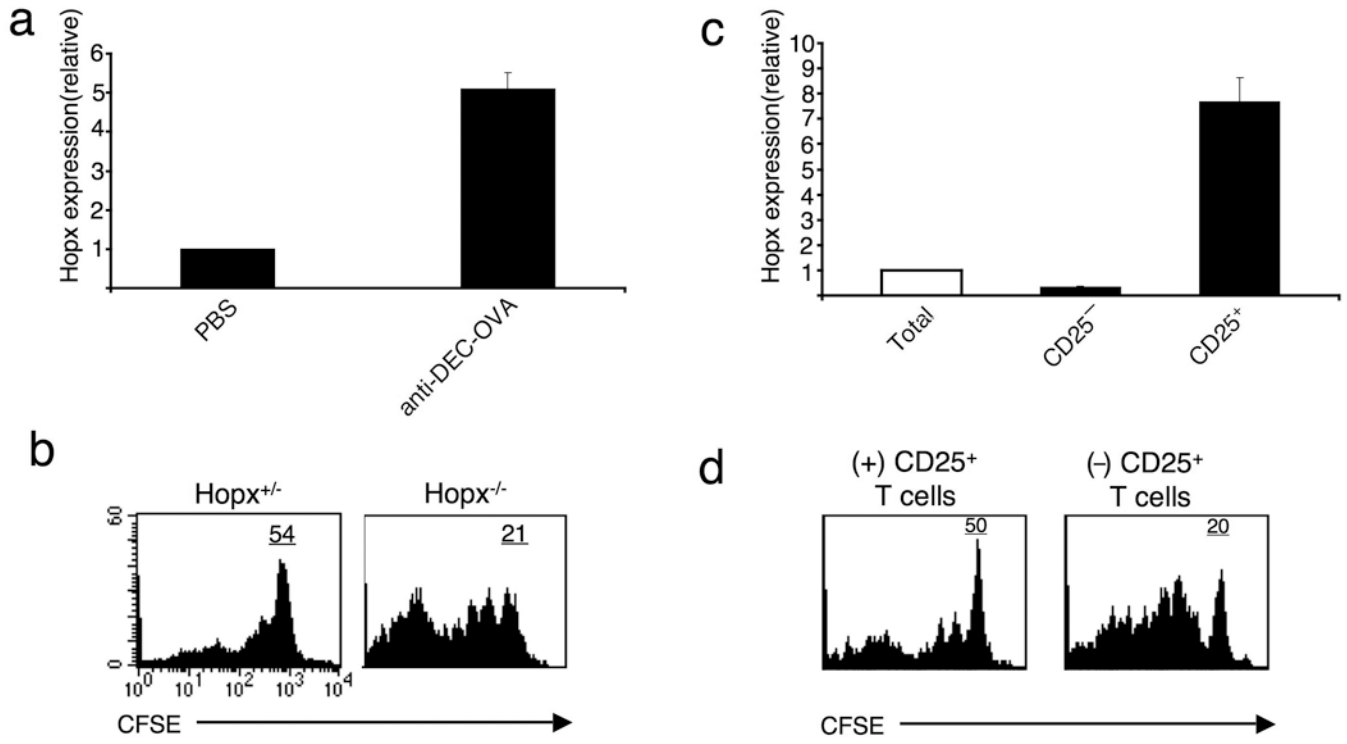

Figure 1. Hopx expressed in iT_{reg} cells is required for T cell unresponsiveness

(**a**) Expression of Hopx is induced during T cell unresponsiveness.

Hopx transcripts were analyzed in CD4 cells from OTII mice pre-treated either with αDEC-OVA or saline control (PBS). Results in each group are measurements (N=3) from pools of 5 separate animals, normalized for expression of HPRT and standardized to *Hopx* expression by the dd CT method. Error bars represent SD. P<0.0008

(**b**) Hopx is required for DC-mediated T cell unresponsiveness.

$Hopx^{-/-}$ and $Hopx^{+/-}$ CD4 cells from αDEC-OVA-pre-treated OTII mice were adoptively transferred as indicated. Recipients were immunized with OVA peptide and T cells were analyzed by flow cytometry 3 days later. Histograms show CFSE intensity and percentages indicate cells that did not proliferate.

The results shown represent one of three similar experiments.

(**c**) Hopx is expressed in iT_{reg} cells induced by DCs.

Hopx gene expression was measured in CD4 cells from αDEC-OVA pre–treated OTII mice as indicated. The results are measurements (N=3) from pools of 8 separate animals normalized for expression of *Hprt* and standardized to *Hopx* expression by the dd CT method. Error bars represent SD. P<0.0002

(**d**) T_{reg} cells induced by DCs are required for T cell unresponsiveness.

$CD4^+CD25^-$ T cells from αDEC-OVA-pre-treated OTII mice were transferred either in absence or presence of $CD4^+CD25^+$ iT_{reg} cells as indicated. Recipients were immunized with OVA peptide and T cells were analyzed by flow cytometry 3 days later. Histograms show CFSE intensity and percentages indicate cells that did not proliferate.

The results shown represent one of three similar experiments.

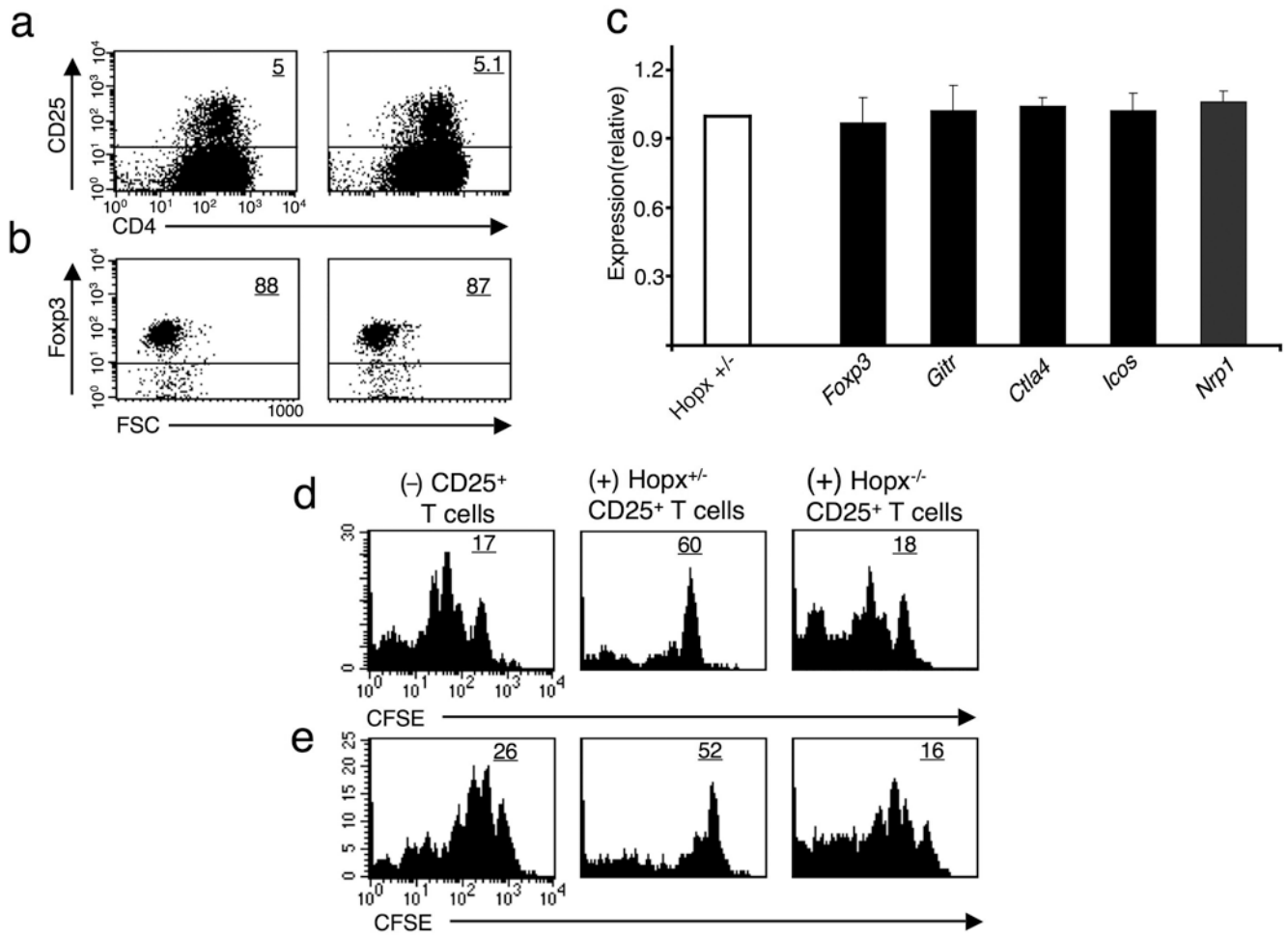

Figure 2. *Hopx*-deficient iT$_{reg}$ cells express T$_{reg}$-specific genes but fail to mediate T cell unresponsiveness *in vivo*

(**a**) iT$_{reg}$ cells are induced by DC in the absence of *Hopx*.

Hopx$^{-/-}$ and *Hopx*$^{+/-}$ CD4 cells from αDEC-OVA-treated mice were analyzed by flow cytometry. The numbers indicate the percentage of double positive CD4^{+}CD25^{+} cells.

(**b**) DC-induced T$_{reg}$s maintain expression of Foxp3 in absence of *Hopx*.

Hopx$^{-/-}$ and *Hopx*$^{+/-}$ CD4^{+}CD25^{+} cells from αDEC-OVA-pre-treated mice were analyzed by flow cytometry. The numbers indicate the percentage of Foxp3 positive cells. The results shown in **a** and **b** represent one of three similar experiments.

(**c**) Comparison of the Foxp3-dependent gene expression in *Hopx*$^{-/-}$ and *Hopx*$^{+/-}$ DC-induced T$_{reg}$ cells.

Gene expression was analyzed by quantitative real-time RT-PCR in *Hopx*$^{-/-}$ and *Hopx*$^{+/-}$ CD4^{+}RFP^{+} cells from αDEC-OVA-pre-treated OTII- FOXP3-IRES-RFP mice. Results in each group (*Hopx*$^{+/-}$ and *Hopx*$^{-/-}$) are measurements (N=3) from pools of 6 separate animals normalized for expression of *Hprt*, and standardized for each gene analyzed to its expression in *Hopx*$^{+/-}$ T$_{reg}$ cells by the dd CT method. Error bars represent SD. P>0.4.

(**d** and **e**) Hopx is required for suppressor function by DC-induced T$_{reg}$s *in vivo*.

Hopx$^{+/-}$ (**d**) or *Hopx*$^{-/-}$ (e) CD4^{+}CD25^{-} T cells from αDEC-OVA-treated OTII mice were transferred either in absence or presence of *Hopx*$^{-/-}$ and *Hopx*$^{+/-}$ DC-induced T_{reg} cells as indicated. Recipients were immunized with OVA peptide and T cells were analyzed by flow cytometry 3 days later. Histograms show CFSE intensity and percentages indicate cells that did not proliferate. The results represent one of three (**d**) and two (**e**) similar independent experiments

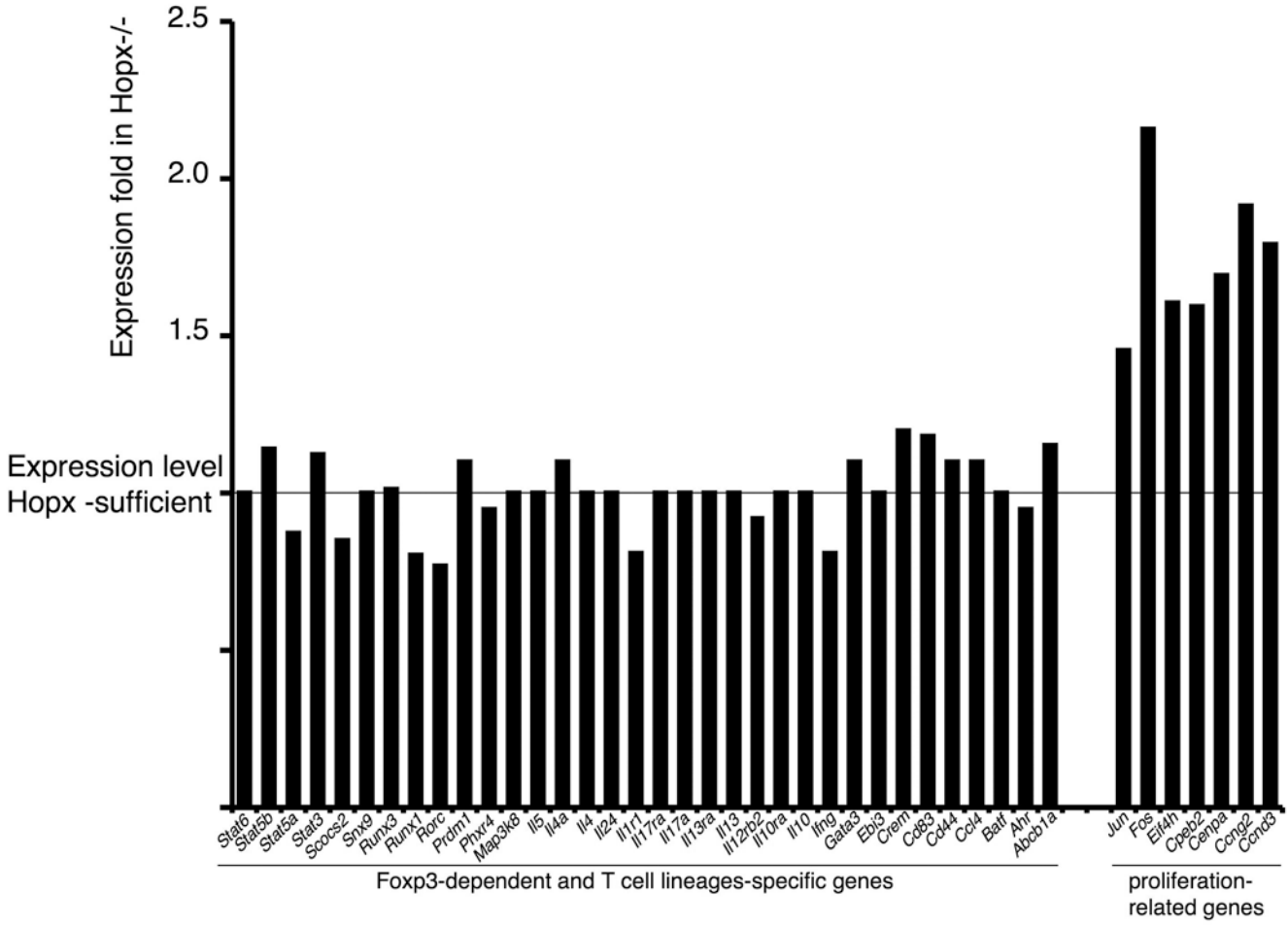

Figure 3. Hopx-dependent expression of proliferation-related genes in DC-induced T_{reg} cells

Hopx$^{-/-}$ and *Hopx*$^{+/-}$ OTII FOXP3-IRES-RFP littermate mice were treated with αDEC-OVA and after 7 days re-challenged by immunization with OVA peptide. TCRα2^{+}CD4^{+}CD25^{+}RFP^{+} (Foxp3^{+}) DC-induced T_{reg} cells were isolated 24h after immunization with OVA in CFA by cell sorting and total gene expression was measured using Affymetrix Mouse Genome 430 2.0 Arrays. Results show change of a particular gene expression in *Hopx*-deficient DC-induced T_{reg} cells compared to its expression level in *Hopx*-sufficient DC-induced T_{reg} cells. 1=expression level in *Hopx* sufficient. Shown are proliferation-related genes (expression altered in absence of *Hopx*) and selected Foxp3-dependent and T cell lineages-specific genes. The results shown are from one of two experiments.

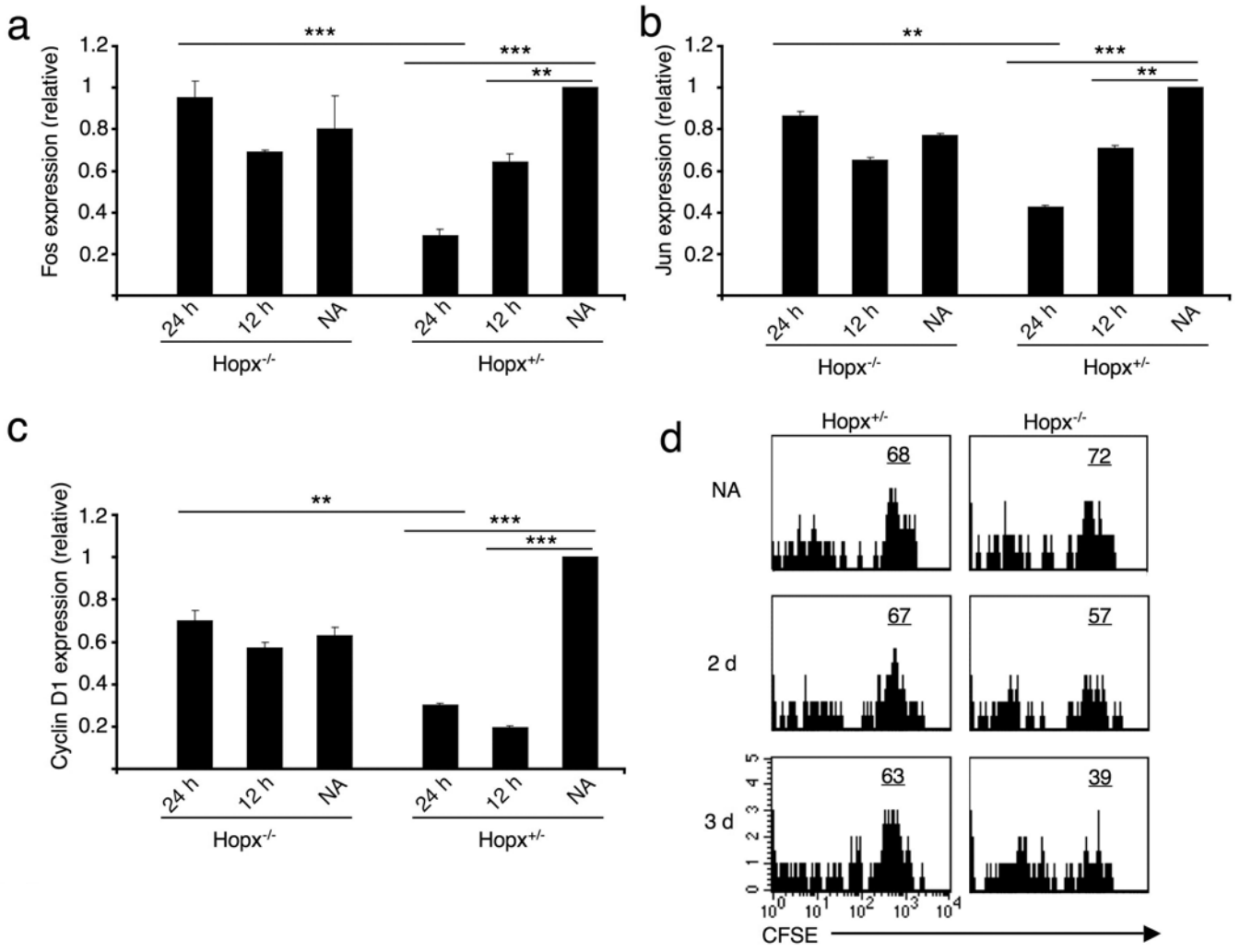

Figure 4. Hopx maintains the state of anergy in DC-induced T_{reg} cells

(**a–c**) Hopx-dependent down-regulation of proliferation-promoting genes in DC-induced T_{reg} cells upon re-challenge with antigen

Hopx$^{-/-}$ and *Hopx*$^{+/-}$ OTII FOXP3-IRES-RFP littermate mice were treated with αDEC-OVA and 7 days later re-challenged by immunization with OVA peptide as in Fig. 3. DC-induced T_{reg} cells were isolated by cell sorting either from αDEC-OVA-treated but un-immunized mice (NA) or 12 or 24h after immunization with OVA as indicated. Transcripts of *Fos* (**a**), *Jun* (**b**) and *Cyclin D1* (**c**) were analyzed by real-time RT-PCR. The results are multiple measurements (N=3) from pools of 6–8 separate animals in each group and are normalized for expression of *Hprt* and standardized by the dd CT method for each gene analyzed to its expression in *Hopx*-sufficient DC-induced T_{reg} cells from mice unimmunized with OVA in CFA. (**P ≤ 0.01 and ***P ≤ 0.001). Error bars represent SD.

(**d**) *Hopx*$^{-/-}$ DC-induced T_{reg} cells proliferate *in vivo* upon re-challenge with antigen

Hopx$^{-/-}$ and *Hopx*$^{+/-}$ OTII FOXP3-IRES-RFP littermate mice were treated with αDEC-OVA and after 7 days DC-induced T_{reg} cells were isolated by cell sorting, labeled with CFSE and transferred into CD45.1^{+} C57BL/6 mice. Recipients were immunized with OVA

peptide or were un-immunized (NA) and T cells were analyzed by flow cytometry 2 or 3 days later. Histograms show CFSE dilution and percentages indicate cells that did not proliferate. The results shown represent one of two similar experiments.

Table 1

Hopx-dependent expression of T cell lineage-specific genes in DC-induced T_{reg} cells

Hopx$^{-/-}$ and *Hopx*$^{+/+}$ OTII FOXP3-IRES-RFP littermate mice were treated with 50 ng of αDEC-OVA and after 7 days re-challenged by immunization with 100 μg OVA peptide in CFA. TCRα2^{+}CD4^{+}CD25^{+}RFP^{+} (Foxp3^{+}) DC-induced T_{reg} cells were isolated 24h after immunization with OVA in CFA by cell sorting and total gene expression was measured using Affymetrix Mouse Genome 430 2.0 Arrays as in Fig. 3. Results show fold-change of a particular gene expression in *Hopx*-deficient DC-induced T_{reg} cells compared to its expression level in *Hopx*-sufficient DC-induced T_{reg} cells of multiple major Foxp3-dependent and T cell lineages-specific genes. 1=expression level in *Hopx* sufficient cells.

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
Abcb1a	1.15	*Ifng*	0.81	*Il21*	1.17	*Pdcd1*	1.17
Ahr	0.95	*Ifngr1*	1.22	*Il21r*	1.03	*Pdcd2*	0.9
Arl6	0.78	*Ifngr2*	1.13	*Il22ra2*	0.85	*Phxr4*	1.05
Atf1	1.01	*Ikzf2*	1.29	*Il23a*	1.08	*Prdm1*	1.13
Batf	1.03	*Il10*	1	*Il24*	1.01	*Prkch*	1.21
Batf3	0.98	*Il10ra*	1.12	*Il2ra*	0.88	*Rhoh*	1.25
Ccl4	1.14	*Il10rb*	1.35	*Il4*	1.04	*Rorc*	0.77
Ccl5	1.26	*Il12rb2*	0.9	*Il4ra*	1.14	*Runx1*	0.8
Ccr6	1.1	*Il13*	1.07	*Il5*	1.02	*Runx3*	1.01
Cd2	1.15	*Il13ra1*	1.06	*Il5ra*	1	*S100a10*	1.21
Cd200	1	*Il17a*	1.02	*Il6ra*	1.2	*Snx9*	1.04
Cd44	1.14	*Il17b*	1.16	*Il9*	1	*Socs2*	0.9
CD83	1.18	*Il17d*	0.77	*Il9r*	0.76	*Stat3*	1.12
Crem	1.25	*Il17ra*	1.15	*Klrg1*	1.17	*Stat4*	1.22
Ctla4	1.26	*Il17rb*	1.28	*Lag3*	0.86	*Stat5a*	0.9
Cxcr4	0.86	*Il17rc*	1.2	*Maf*	0.86	*Stat5b*	1.14
Ebi3	1.06	*Il17rd*	1.01	*Map3k8*	1.05	*Stat6*	1.01
Foxp3	1.21	*Il17re*	1.02	*Notch1*	1	*Tbc1d4*	1.29
Gata3	1.11	*Il1r1*	0.81	*Notch2*	1	*Tbx21*	0.7
Gitr	0.97	*Il1r1*	0.81	*P4ha1*	1.28	*Tgfbr1*	1.19

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
Icos	1.26	*Il1r2*	0.92	*P4ha1*	1.28	*Tgfbr2*	1.25