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Dynamic Interactions between TIP60 and p300 Regulate FOXP3 Function through a Structural Switch Defined by a Single Lysine on TIP60

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SUMMARY

The human FOXP3 molecule is an oligomeric transcriptional factor able to mediate activities that characterize T regulatory cells, a class of lymphocytes central to the regulation of immune responses. The activity of FOXP3 is regulated at the posttranslational level, in part by two histone acetyltransferases (HATs): TIP60 and p300. TIP60 and p300 work cooperatively to regulate FOXP3 activity. Initially, p300 and TIP60 interactions lead to the activation of TIP60 and facilitate acetylation of K327 of TIP60, which functions as a molecular switch to allow TIP60 to change binding partners. Subsequently, p300 is released from this complex, and TIP60 interacts with and acetylates FOXP3. Maximal induction of FOXP3 activities is observed when both p300 and TIP60 are able to undergo cooperative interactions. Conditional knockout of TIP60 in Treg cells significantly decreases the Treg population in the peripheral immune organs, leading to a scurfy-like fatal autoimmune disease.

INTRODUCTION

FOXP3 plays an important role in the regulation of Treg function (Fontenot et al., 2003; Hori et al., 2003; Li and Greene, 2007). Acetylation, a process catalyzed by opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs), is one set of posttranslational modifications that regulates the stability and transcriptional activity of FOXP3. HATs and HDACs were first identified as enzymes responsible for histone acetylation but were later found to promote acetylation of many substrates other than histone (Li et al., 2007; Tao et al., 2007; van Loosdregt et al., 2010; Xiao et al., 2010; Zhang et al., 2012).

Based on sequence homology, HATs can be divided into three major categories: the Gcn5/PCAF family, p300/CBP family, and MYST family (Yang, 2004). Two HATs, TIP60, a member of the MYST family, and p300, of the p300/CBP family, have been reported to promote FOXP3 acetylation (Li et al., 2007; Liu et al., 2013; van Loosdregt et al., 2010). TIP60 interacts with the N-terminal domain of FOXP3 and is required for the increased repressive transcriptional activity of FOXP3. Acetylation of lysine (K) 8 of FOXP3 promoted by TIP60 is important to the increased activity of FOXP3 because a HAT-deficient TIP60 mutant is not able to enhance pFOXP3-suppressive activity (Li et al., 2007). p300 has been suggested to have a similar effect in promoting the repressive transcriptional activity of FOXP3 by increasing the stability of certain pools of FOXP3 (van Loosdregt et al., 2010). As in the case of many other proteins, the stability of FOXP3 is regulated by ubiquitination, which leads to proteosome-mediated protein degradation. The p300 moiety increases the acetylation level of FOXP3, which then decreases the ubiquitination level of FOXP3, preventing its degradation (van Loosdregt et al., 2011).

In a comparable manner to regulation of the activity of many kinases by phosphorylation, the acetyltransferase activities of certain HATs are also regulated through acetylation catalyzed either by itself or by other HATs. Autoacetylation of TIP60 can be induced by diverse signals such as UV irradiation of cells. This type of injury and its signals increase TIP60 HAT activity. Deacetylation of TIP60 by Sirtuin 1 (SIRT1) decreases its HAT activity and maintains levels of TIP60 proteins (Wang and Chen, 2010; Yamagata and Kitabayashi, 2009). Similarly, autoacetylation is also important for the function of p300. Autoacetylation of an inhibitory loop in p300 is thought to be required to activate the HAT activity of p300 and increase substrate accessibility (Thompson et al., 2004). p300 may further promote the acetylation of TIP60 (Col et al., 2005). Therefore, a complicated set of interactions occurs between different HATs and is required for regulation of acetyltransferase activities.

TIP60 and p300 have been identified previously as HATs that individually influence the activity of FOXP3 (Li et al., 2007; Liu et al., 2013; van Loosdregt et al., 2010). Because acetylation is critical to the function of FOXP3, understanding the separate and combined roles of these HATs in the regulation of FOXP3 is important to understand the molecular mechanisms involved in regulation of Treg cells.

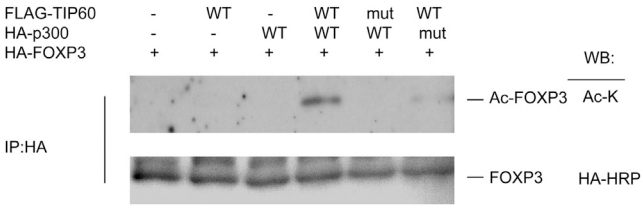


Figure 1. Cooperative Effect of TIP60 and p300 on FOXP3 Acetylation

293T cells are transfected with HA-FOXP3, FLAG-TIP60, FLAG-p300, or HAT-deficient FLAG-TIP60 mutant (mut) or FLAG-p300 mutant as indicated. Twenty-four hours after transfection, cell lysates were collected and immunoprecipitated (IP) with anti-HA agarose, followed by blotting with anti-acetylated lysine antibody or anti-HA antibody. WB, western blot.

Our studies indicate that p300 interactions with Tip60 promote TIP60 autoacetylation, which we have defined as important to maintain the stability of the TIP60 protein. p300 interaction with Tip60 also critically promotes a specific modification that acts as a switch to govern TIP60's interaction with its substrates. TIP60 in turn promotes p300 acetylation that is critical for HAT activity of p300. Thus, these two enzymes promote the acetylation level and HAT activities of each other, which promote a synergistic effect on FOXP3 acetylation and increase the repressive transcriptional activity of FOXP3. We have also unexpectedly discovered a dominant role for TIP60 in maintenance of peripheral Treg survival and function. Selective loss of TIP60 in FOXP3-expressing Treg cells can lead to significant peripheral deficits of suppressive activity that lead to catastrophic scurfy-like disease.

RESULTS

TIP60 and p300 Promote FOXP3 Acetylation Cooperatively in a HAT-Dependent Manner

Both TIP60 and p300 have been shown to promote the acetylation of FOXP3. We sought to understand if these enzymes act in a cooperative manner. To investigate this cooperative interplay, 293T cells were cotransfected with FOXP3, TIP60, and p300. The acetylation of FOXP3 was then determined in the presence of TIP60 and p300. Figure 1 shows that a strong acetylation pattern of FOXP3 is observed when both TIP60 and p300 are present. In the absence of either enzyme, however, acetylation of FOXP3 is weak. These studies indicate that synergistic interactions occur between TIP60 and p300 in dominant acetylation of FOXP3.

We next evaluated the contributory role of intrinsic TIP60 HAT activity for the coactivation of p300 and other transcription factors (Korzus et al., 1998; Senf et al., 2011). The acetylation of FOXP3 was tested using HAT-deficient TIP60 (Q377E/G380E) and p300 (F1504A) (Ikura et al., 2000; Ito et al., 2001) species. Although the acetylation of FOXP3 was strong in the presence of both wild-type (WT) TIP60 and WT p300, no acetylation was observed when either of the HAT-deficient mutants was present (Figure 1). These studies indicate that the cooperative effects that occur between TIP60 and p300 are HAT dependent with respect to acetylation of FOXP3.

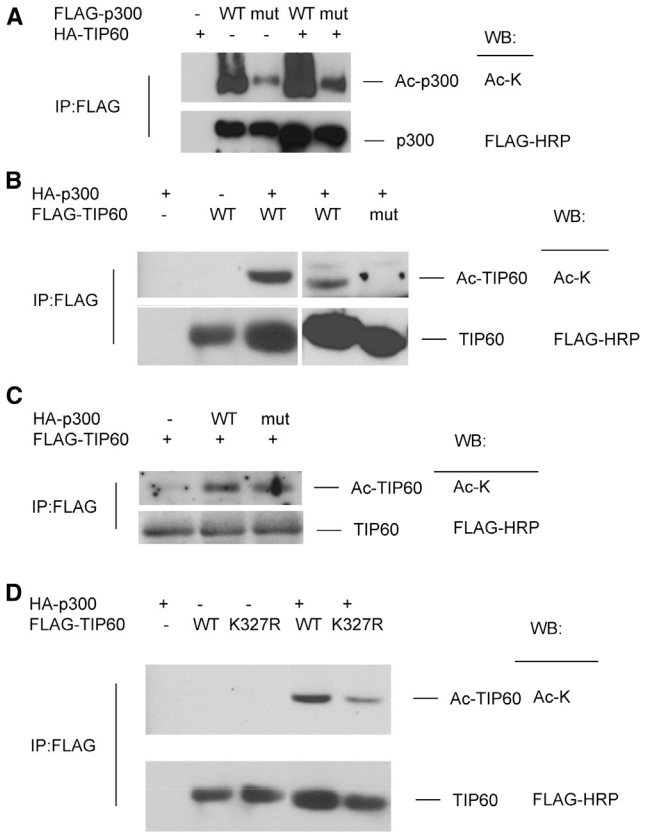


Figure 2. p300 and TIP60 Promote the Acetylation of Each Other

(A) TIP60 promotes the acetylation of p300. 293T cells were transfected with HA-TIP60 and WT or HAT-deficient FLAG-p300 as indicated. Twenty-four hours after transfection, cell lysates were immunoprecipitated with anti-FLAG agarose and blotted with anti-acetylated lysine or anti-FLAG HRP.

(B–D) 293T cells were transfected with HA-p300 and WT or mutated FLAG-TIP60 as indicated. Twenty-four hours after transfection, cell lysates were immunoprecipitated with anti-FLAG agarose and blotted with anti-acetylated lysine antibody or anti-FLAG HRP.

(B) p300 promotes the acetylation level of WT TIP60, but not HAT-deficient TIP60 mutant.

(C) HAT-deficient p300 mutant has the same effect in promoting TIP60 acetylation.

(D) TIP60 K327R mutation decreases TIP60 acetylation.

p300 Acetylation Promoted by TIP60 Requires HAT Activity of TIP60

Because TIP60 and p300 work synergistically to promote the acetylation of FOXP3, we investigated how each enzyme is affected by the activity of the other. To study this, the acetylation level of p300 was tested in both the presence and absence of TIP60. As shown in Figure 2A, the presence of TIP60 led to increased acetylation of p300. Surprisingly, this modification effect was even more significant for the HAT-deficient mutant of p300. Because the p300F1504A mutant lacks the HAT activity required for autoacetylation, the increased level of acetylation of p300 must be induced by the HAT activity of TIP60. In addition, the acetylation of p300 may affect how it binds to other proteins (Thompson et al., 2004). Therefore,

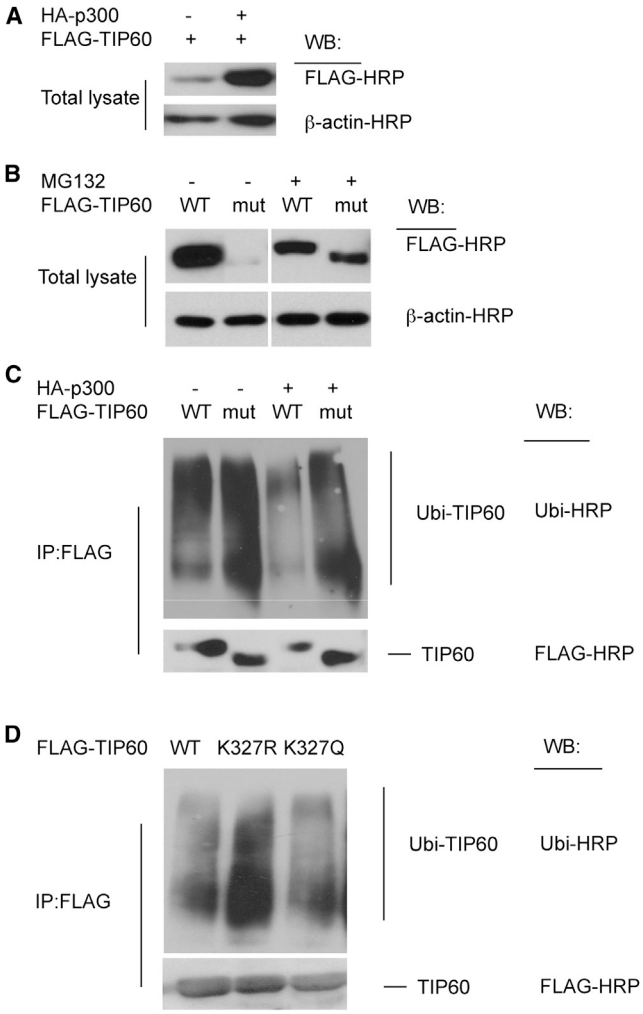


Figure 3. TIP60 Acetylation Increases TIP60 Stability through Inhibiting TIP60 Ubiquitination

(A) Expression level of TIP60 is significantly increased in the presence of p300. 293T cells were transfected with FLAG-TIP60 alone, or cotransfected with FLAG-TIP60 and HA-p300. Twenty-four hours after transfection, cell lysates were collected and blotted with anti-FLAG HRP or anti-β-actin HRP.

(B) Expression level of TIP60 HAT-deficient mutant can be increased by MG132 treatment. 293T cells were transfected with WT or HAT-deficient FLAG-TIP60 mutant. Twenty-four hours after transfection, cells were treated with 2 μM MG132 for 16 hr. Cell lysates were then collected and blotted with anti-FLAG HRP or anti-β-actin HRP.

(C and D) TIP60 ubiquitination is correlated with TIP60 acetylation. 293T cells were cotransfected with HA-p300 and WT or HAT-deficient FLAG-TIP60 mutant (C), or transfected with WT FLAG-TIP60 or FLAG-TIP60 K327 mutants (D). Twenty-four hours after transfection, cells were treated with 2 μM MG132 to prevent proteasome-dependent degradation of ubiquitinated TIP60. Cell lysates were immunoprecipitated with anti-FLAG HRP and blotted with anti-ubiquitin HRP or anti-FLAG HRP.

we examined the interaction between TIP60 and p300 and unexpectedly discovered reduced interactions between the HAT-deficient p300 mutant and TIP60 (Figure S1), indicating that acetylation activity of p300 correlates with its ability to interact with TIP60.

TIP60 Autoacetylation at K327 Is Promoted by Its Interaction with p300

It was unclear whether the HAT activity of both enzymes is required for p300 to promote TIP60 acetylation. Therefore, HAT-deficient mutants of TIP60 and p300 were employed to resolve this issue. Although WT p300 significantly increased the acetylation of WT TIP60, it had no effect on the TIP60 mutant (Figure 2B), indicating that TIP60 relies on its own intrinsic autoacetylation even in the presence of p300. The p300 mutant, on the other hand, showed comparable activity to that of WT p300 to enhance TIP60 acetylation (Figure 2C). Thus, unlike TIP60, the HAT activity of p300 does not play a determinant role in promoting TIP60 acetylation.

K327 of TIP60 has been identified as a strictly conserved lysine site among the MYST family proteins, and moreover, this residue can be autoacetylated (Peng et al., 2012; Wang and Chen, 2010; Yang et al., 2012). We explored the possibility that autoacetylation at K327 is promoted by p300 collisions as well. In the absence of p300, autoacetylation of TIP60 is totally abolished by the K327R mutation, indicating that K327 is the major autoacetylation site in TIP60 (Figure S2). Similarly, in the presence of p300, the acetylation of TIP60 was significantly reduced when K327 was substituted with arginine (Figure 2D), indicating that p300 physical interactions with Tip60 actually promote the autoacetylation of TIP60 at K327. Acetylation of TIP60 (in particular autoacetylation) is known to be important for supporting the total HAT activity of TIP60 (Yang, 2004). Therefore, K327 acetylation promoted by p300 also regulates the activity of TIP60.

However, unexpectedly mutating K327 in TIP60 only slightly decreased the cooperative effect of TIP60 and p300 in promoting Foxp3 acetylation. Our studies thus distinguish the consequences of two discreet mutations. Unlike the Q377E/G380E mutation of TIP60 that limits HAT activity and FOXP3 modification, the TIP60 K327 residue is not critical for the cooperative effects of TIP60 and p300 that lead to cooperative FOXP3 acetylation (Figure S3).

Autoacetylation of TIP60 Promoted by p300 Increases TIP60 Stability through Inhibiting TIP60 Ubiquitination

Posttranslational acetylation is important for protein stability because it prevents protein degradation mediated by ubiquitination (Caron et al., 2005). To investigate whether p300 regulates TIP60 in a similar manner, the expression level of TIP60 was examined. Expression of TIP60 was found to be increased in the presence of p300 (Figure 3A). To define the correlation between acetylation and protein stability, the HAT-deficient mutant of TIP60 was studied. As shown in Figure 3B, expression of the TIP60 mutant was significantly reduced compared to that of WT TIP60, indicating the crucial role acetylation plays in the stability of the TIP60 protein. Only in the presence of MG132, a chemical used to inhibit proteasome-dependent protein degradation, was the expression level of the TIP60 mutant restored (Figure 3B).

We further investigated the ubiquitination patterns of TIP60. Ubiquitination of WT TIP60 was much lower than that of the HAT-deficient mutant (Figure 3C). In addition, the presence of p300 further decreased ubiquitination of WT TIP60 due to increased acetylation promoted by p300. Because p300

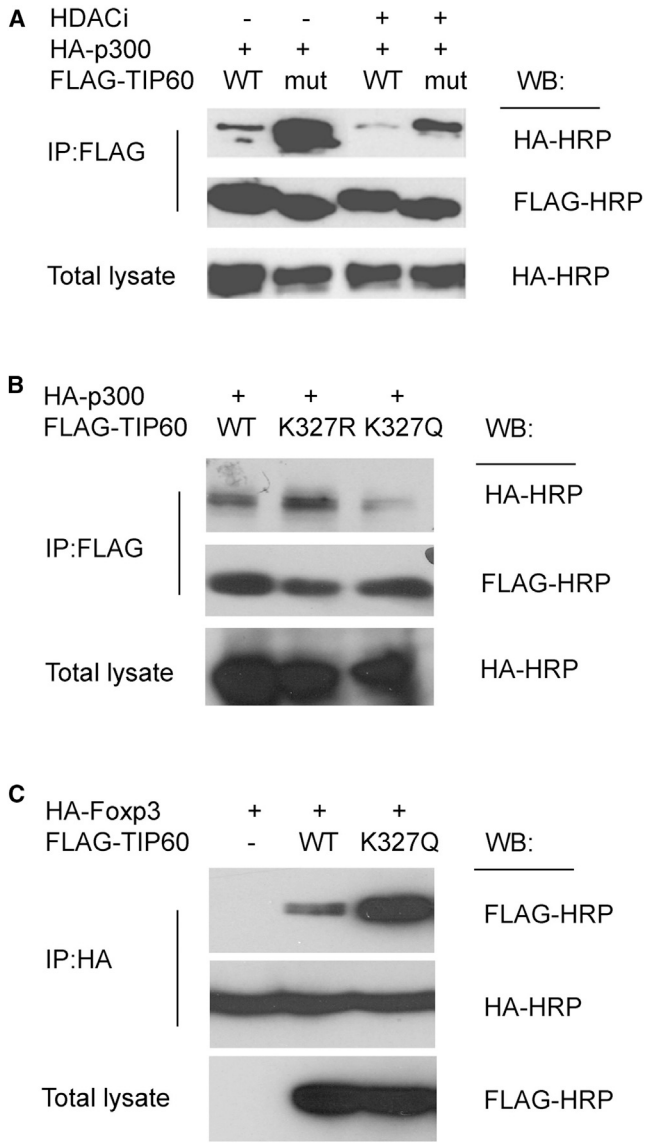


Figure 4. Interaction of TIP60 with Its Substrates Is Regulated by the Acetylation Status of TIP60

(A) Reverse correlation between TIP60 acetylation and interaction of Tip60 and p300. 293T cells were transfected with HA-p300, WT, or HAT-deficient FLAG-TIP60. Twenty-four hours after transfection, cells were treated with 400 nM Trichostatin A (TSA) and 10 mM nicotinamide (NAD) for 16 hr. Cell lysate was then immunoprecipitated with anti-FLAG agarose and blotted with anti-HA HRP or anti-FLAG HRP.

(B) Acetylation status at TIP60 K327 regulates the interaction of TIP60 and p300. 293T cells were transfected with HA-p300 and WT FLAG-TIP60 or FLAG-TIP60 K327 mutants. Twenty-four hours after transfection, cell lysate was immunoprecipitated with anti-FLAG agarose and blotted with anti-HA HRP or anti-FLAG HRP.

(C) TIP60 acetylation at K327 increases the interaction of TIP60 and FOXP3. 293T cells were cotransfected with HA-FOXP3 and WT FLAG-TIP60 or FLAG-TIP60 K327Q mutant. Twenty-four hours after transfection, cell lysate was immunoprecipitated with anti-HA agarose and blotted with anti-FLAG HRP or anti-HA HRP.

promotes the autoacetylation of TIP60 at K327, we also studied the role of K327 in regulating the ubiquitination of TIP60. When we substituted lysine with arginine, the K327R TIP60 mutant showed reduced autoacetylation (data not shown) but increased ubiquitination as compared with WT TIP60 (Figure 3D).

It is notable that a decrease in ubiquitination occurred when K327 was mutated to glutamine, a modification that mimics acetylation at this site. These results indicate that K327 is not a ubiquitination site itself but rather that the acetylation of this site promotes a conformational change of TIP60 to prevent ubiquitination of the protein. Together, our data indicate that the autoacetylation of TIP60 on K327, which can be enhanced by p300, limits the protein from ubiquitination-mediated proteasomal degradation by a mechanism that apparently relates to large-scale changes in protein conformation.

Acetylation Regulates the Interaction of TIP60 with p300 and FOXP3

We extended these studies to examine if autoacetylation of K327 of the TIP60 acetyltransferase promoted substrate switching. Acetylation may regulate protein function by altering protein-protein interactions, and acetylation of human ortholog of MOF (hMOF) at K274 changes the spatial orientation of that particular lysine, altering the interactions of hMOF with certain substrates (Yuan et al., 2012). Further autoacetylation of TIP60 may dissociate the TIP60 oligomer, resulting in the activation of TIP60. Autoacetylation-driven dissociation may further lead to an improved accessibility for its substrates (Wang and Chen, 2010). We examined the interactions of TIP60 with either p300 or FOXP3.

Surprisingly, our results showed that WT TIP60 interacted much more weakly with p300 than the HAT-deficient mutant of TIP60 (Figure 4A). This interaction was further decreased by HDAC inhibitor (HDACi) treatment that prevents the deacetylation of TIP60. Together, these studies identify that autoacetylation lessens the interactions of TIP60 with other proteins such as p300.

To determine if acetylation of the K327 residue of TIP60 itself influences the interactions of TIP60 with other proteins, we first investigated the interactions between p300 and the K327 mutants of TIP60 (Figure 4B). The K327R mutant of TIP60, which is less acetylated than the WT species, interacted strongly with p300, whereas the K327Q mutant, a residue mutation that mimics the acetylated status of WT K327, was found to interact only weakly with p300.

Thereafter, we investigated whether the interactions between FOXP3 and TIP60 are altered by the acetylation of TIP60. Because the autoacetylation of TIP60 is weak in the absence of p300, the K327Q mutant was used to mimic the acetylated TIP60. In contrast to the interaction of TIP60 and p300, a much stronger interaction with FOXP3 was observed for the TIP60 K327Q mutant as compared with the WT TIP60 (Figure 4C). In vitro pull-down assays using purified TIP60 and Foxp3 fragments also verified that TIP70 K327R mutants have stronger interaction with Foxp3 (Figure S4A). The same results were also observed when other substrates of TIP60 are used, such as p53 or HDAC7 (Figures S4B and S4C). Collectively, our data indicate that the acetylation of the single K327 residue plays a pivotal role in the regulation of the interactions of TIP60 with

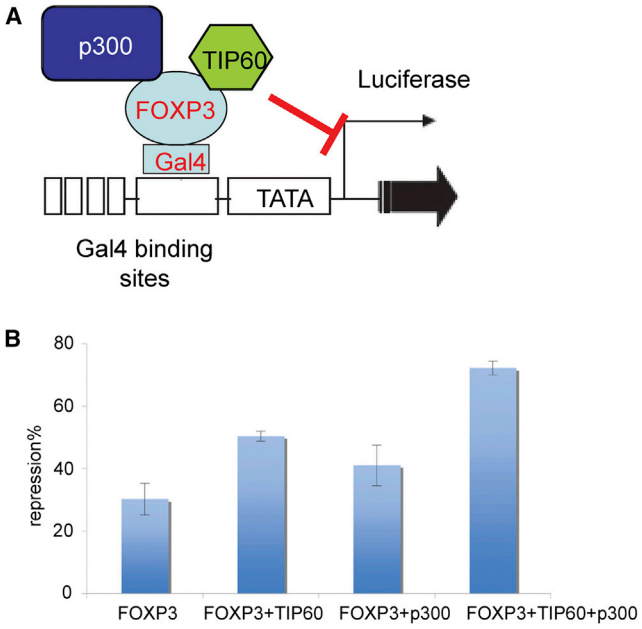


Figure 5. p300 and TIP60 Increase the Repressive Transcriptional Activity of FOXP3 Synergistically

(A) Schematic model of FOXP3 binding to luciferase reporter construct used in luciferase assay.

(B) Synergistic effect of TIP60 and p300 on the repressive transcriptional activity of FOXP3. 293T cells were transfected with pBIND (empty vector), pBIND-FOXP3, FLAG-TIP60, FLAG-p300, pG5-Luc luciferase reporter, and the control MSV-β-gal plasmid as indicated. The luciferase activity of the reporter gene was normalized with β-gal activity. The error bars indicate the SD value.

other proteins and provide a biochemical explanation for increased interactions with FOXP3 after substrate-switching conformations are induced.

Transcriptional Activity of FOXP3 Is Promoted by TIP60 and p300 Cooperative Interactions

As mentioned, TIP60 and p300 can cooperatively increase the acetylation of FOXP3. We examined if cooperative interactions between TIP60 and p300 that affect FOXP3 acetylation correlate with the change in the transcriptional activity of FOXP3. A facile transcriptional repression assay has been established to determine the effect of FOXP3, using a Gal4-FOXP3 fusion protein, on the expression of the firefly luciferase reporter gene driven by a promoter region containing five Gal4-binding sites (Li et al., 2007). This system was used to evaluate the cooperative effect of TIP60 and p300 on the transcriptional activity of FOXP3 (Figure 5A). The MSV-β-gal vector with a constitutive expression of β-galactosidase (β-gal) was used as a control for transfection. As shown in Figure 5B, FOXP3 alone repressed transcription of the luciferase reporter gene. Such repression was slightly enhanced in the presence of either TIP60 or p300. When both enzymes were present, however, there was a significant increase in the repressive transcriptional activity of FOXP3, indicating that TIP60 and p300 function cooperatively to increase the transcriptional activity of FOXP3.

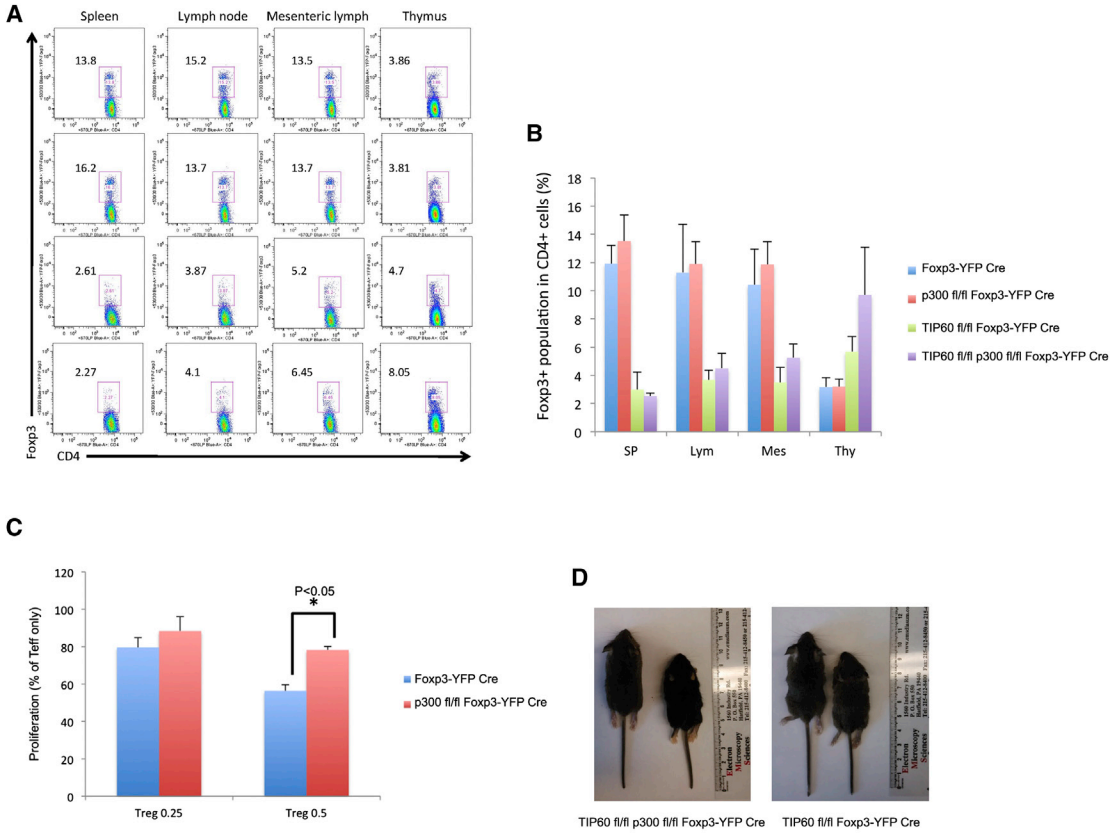
Effect of p300 and TIP60 in the Development of Treg Cells

To define the importance of TIP60 and p300 in regulating Treg function in vivo, we conditionally deleted p300 or TIP60 in Treg cells by crossing $p300^{fl/fl}$ or $TIP60^{fl/fl}$ mice with $Foxp3^{YFP\text{-}Cre}$ mice. The resultant $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice were further crossed with $TIP60^{fl/fl}$ mice to generate $p300^{fl/fl}$ $TIP60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice, which represent double conditional knockouts of p300 and TIP60 in Treg cells.

$p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice developed normally until 8 weeks of age with a normal population of Treg cells in thymus, lymph node, and spleen (Figures 6A and 6B). The size of $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice is also similar to the littermate control without Foxp3-Cre gene (data not shown). Although p300 is important for the stability of the Foxp3 protein, suppressive assays using Treg cells from $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice reveal that p300 has a modest effect on the suppressive function of Treg cells (Figure 6C). In accordance with this, $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice have larger spleen and lymph nodes compared with $Foxp3^{YFP\text{-}Cre}$ mice, but the size of lymph nodes from $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ is smaller than that from $Tip60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ or $p300^{fl/fl}$ $TIP60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice (Figure S5A). Hematoxylin and eosin (H&E) staining of liver and lung sections also shows modest inflammation in these sections (Figure S5B). p300 therefore plays a role in regulating the development and function of Treg cells, but it is not absolutely required to prevent the development of fatal autoimmune diseases.

In contrast to $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice, $Tip60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ and $p300^{fl/fl}$ $TIP60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice developed severe weight loss, dermatitis, and splenomegaly from 2 weeks old and died at an early age (Figures 6D and S5). Treg cells from these mice were analyzed to investigate the role of TIP60 in regulating Treg function. Surprisingly, TIP60 knockout in Foxp3-expressing cells greatly decreased the Treg populations in both the spleen and lymph node (Figures 6A and 6B), indicating an indispensable role of TIP60 in the peripheral development and function of Treg cells. Unexpectedly, the Treg population in the thymus is increased by TIP60 knockout, or in the TIP60 and p300 double knockout, indicating that TIP60 is differentially required for the development of Treg cells in thymus or in periphery. TIP60 knockout in Treg cells might cause a defect in the exit of Treg cells from thymus, and TIP60-influenced functionalities are important in peripheral aspects of Treg cell biology.

The suppressive function of Tregs from $Tip60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ and $p300^{fl/fl}$ $TIP60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice could not be characterized due to the paucity of Treg cells in spleen and lymph node. CD4+ T cells acquire suppressive function when they are transduced with Foxp3. Therefore, to investigate the role of TIP60 in regulating the suppressive function of Treg cells, CD4+ naive T cells were transduced with both Foxp3 and WT TIP60 or TIP60 mutants, then the suppressive function of these transduced cells was investigated. As expected and as reported previously, TIP60 HAT-deficient mutants (Q377E/G380E) show reduced suppressive function compared to WT TIP60 (Figure 6D). However, we note that the TIP60 K327Q mutant-transduced T cells yield intermediate suppression. Structurally, whereas K327Q mimics the



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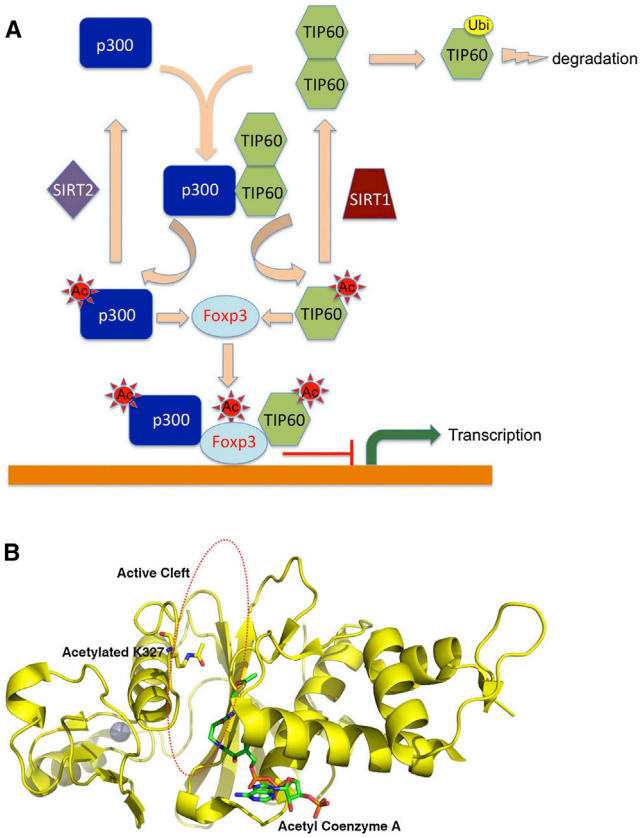

Figure 7. Schematic Model of the Cooperation between TIP60 and p300 on the Regulation of FOXP3

(A) Cooperative actions of TIP60 and p300. TIP60 and p300 promote the acetylation of each other, and their deacetylation is catalyzed by SIRT1 and SIRT2, respectively. Deacetylated TIP60 is ubiquitinated and then degraded in a proteasome-dependent manner, whereas acetylated TIP60 disassociates with p300 and forms a stable complex with FOXP3. Together with p300, TIP60 promotes the acetylation of FOXP3, and the repressive transcriptional activity of FOXP3 is maximized by the synergistic effect of TIP60 and p300.

(B) Structural model of acetylated TIP60 indicating that acetylation of K327 would favor FOXP3 binding to the cleft.

conformation of acetylated 327, it does not permit the entire set of functions promoted by acetylation of that specific residue in TIP60. Complete functionality may require further conformational changes after acetylation that induce more consistent substrate switching and binding. Overall, these data indicate that TIP60 is indispensable for the development and function of a population of peripheral Treg cells.

DISCUSSION

The acetylation of lysine residues represents an important post-translational modification to modify the activity of transcriptional factors. During our studies of FOXP3 complexes, we observed that two important HATs, TIP60 and p300, interact cooperatively to promote the acetylation of FOXP3. Based on these findings, we also investigated the molecular mechanisms responsible for this cooperative effort. Our results indicate that the acetylation of TIP60 and p300 triggers a specific residue-defined "switch" that is responsible for controlling the TIP60 protein stability, HAT activity, and substrate interactions. Acetylation of K327 is proposed to alter conformation to promote changes in substrate interactions. We have developed a scheme that illustrates this complex process and also created a structural model to identify the atomic features of Tip60 that appear relevant (see Figures 7A and 7B).

Our results also indicate that the cooperative interactions between TIP60 and p300 lead to a complex mode of regulation for FOXP3. Acetylation of the p300 molecule increases the enzyme's HAT activity (Thompson et al., 2004). TIP60 regulates FOXP3 indirectly by mediating the acetylation of p300. With increased acetylation, p300 can then acetylate FOXP3 at K249 and K251, forcing an atomically definable structural change in FOXP3's dimers (Song et al., 2012). At the same time, p300 regulates TIP60 modifications as well. p300 interacts with the zinc finger region of TIP60 to promote its acetylation (Col et al., 2005). Our studies now identify molecular details of the process by which p300 regulates TIP60. Using the HAT-deficient mutant of TIP60 in the presence of p300, we demonstrated that intrinsic autoacetylation processes are responsible for the increased acetylation of TIP60 that results from this interaction.

There are three different paths by which the acetylation process regulates TIP60. First, acetylation of K327 of TIP60 is important for the HAT activity of TIP60. Therefore, K327 acetylation promoted by p300 interactions enhances the HAT activity of TIP60. Second, acetylation increases the stability of TIP60 by inhibiting ubiquitination, thus preventing proteasome-dependent degradation. When compared to the HAT-deficient mutant of TIP60, WT TIP60 showed less ubiquitination and, therefore, increased expression. Upon interaction with p300, ubiquitination of TIP60 was further decreased. Third, acetylation regulates TIP60's interactions with other proteins. Oligomerization of TIP60 is disrupted by acetylation, which can be reversed by SIRT1 (Wang and Chen, 2010). In this study, we showed that the acetylation of TIP60 at one residue, K327, prompted a protein conformation change that leads to disassociation from p300 along with the reassociation with its substrates such as

Figure 6. TIP60 Plays a Major Role in the Maintenance of Peripheral Treg Cells

(A) Representative dot plots of Treg populations in thymus, lymph node, mesenteric lymph node, and spleen from $Foxp3^{YFP\text{-}Cre}$, $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$, $Tip60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$, and $p300^{fl/fl}$ $TIP60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice.

(B) The average percentage of CD4+ T cells expressing Foxp3 in thymus, lymph node, mesenteric lymph node, and spleen. The percentages shown are the mean values from three individual mice. The error bars indicate the SD value.

(C) Suppressive function of Treg cells from $p300^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice.

(D) Size of TIP60 conditional knockout mouse. Left is littermate without Foxp3-cre gene.

(E) CD4+ T cells were transduced with Foxp3 and WT TIP60 or TIP60 mutant. Transduced T cells were then collected and subject to suppressive assay at the indicated Treg and Teff ratio.

FOXP3. Of note, our laboratory has recently identified small allosteric molecules that target TIP60 functions. These synthetic allosteric small molecules can promote Treg functionalities (unpublished data) supporting the role of changing TIP60 conformation to alter function.

We now propose a schematic model of how p300 and TIP60 cooperatively regulate FOXP3 functionalities (Figure 7A). To begin with, both p300 and SIRT1 regulate the function of TIP60 by modulating its acetylation. With SIRT1, deacetylation of TIP60 occurs, resulting in the formation of an oligomeric complex. Upon its interaction with p300, acetylation of TIP60 then occurs. Once acetylated, TIP60 dissociates from the oligomer as well as p300 to facilitate its final interaction with FOXP3.

Although the role of TIP60 and p300 in regulating Foxp3 activity has herein been delineated in vitro, relatively little is known about the roles of these discreet enzymes in regulating the function of Treg cells. The complex role of p300 includes maintaining Treg/Th17 cell levels by contributing to differentiation events of Th17 cells (Dang et al., 2011). Although p300 has been shown to be important to Foxp3 activity in vitro, a dominant regulatory significance of p300 was not observed in this study. We are aware that we have not excluded other HAT enzymes with similar functions to p300, such as CBP. CBP is highly homologous to p300 and shares many common substrates including FOXP3 (M.I.G. and Y.X., unpublished data). Consequently, we conclude that it is the redundancy of the p300 type of HAT that accounts for its role in vivo, and disabling p300 by itself may not suffice to alter Treg activities.

By conditionally knocking out TIP60 in Treg cells, we show that TIP60 plays a vital role in some fundamental requirement of Treg cells in the periphery. The decrease of Treg population may result from increased CD4+ T cell numbers due to impaired Treg function and T cell activation. However, Treg is not the population that develops independently of CD4+ cells. The observation that the ratio of Foxp3+ cells is decreased indicates that there is a problem in the generation of Treg population in these mutant mice. We consider that migration or some survival function of Treg cells in the periphery may be a reason that the $Tip60^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$ mice develop fatal autoimmune diseases similar to those seen in scurfy mice.

Our data indicate that TIP60 is an important enzymatic factor that is differentially required for the survival of thymic and peripheral Treg cells. Foxp3 forms dynamic complexes with other proteins under different stimulatory conditions. It is noteworthy that Treg-specific elimination of NFAT2, which is part of the Foxp3 complex, has been found to limit peripheral follicular regulatory T cell (T_{FR}) populations possibly due to impaired homing to B cell follicles (Li et al., 2007; Vaeth et al., 2014). We suggest that TIP60 is also present in a vital complex that is specially required for the survival of peripheral Treg cells and whose disruption leads to fatal autoimmune diseases.

Recent studies also indicate nonenzymatic roles of deacetylases (HDAC3) in cellular functions (Sun et al., 2013), whereby physical interactions of such vital complexes guide some functions. In this regard, binding of HDAC6, independent of its deacetylase activity, may contribute to regulation of TIP60 target genes (Chen et al., 2013). Thus, TIP60 may also possess HAT-independent activities that can lead to different functional outcomes. Investigations using mice with dominant-negative TIP60 forms that lack HAT activity but retain substrate-interaction surfaces needed to form dynamic complexes with other proteins will be informative and are underway.

TIP60 and p300 are members of two important and structurally distinct HAT families that regulate many aspects of cellular function. We have discovered some of the features of how these two distinct family elements cooperate. Our study demonstrates the critical role of TIP60 in maintaining peripheral Treg cells and limiting autoimmune responses, therefore providing a new target for regulating immune responses therapeutically. We have also defined a correlation between autoacetylation and ubiquitination of TIP60 that provides further mechanistic insight into the regulation of TIP60's activity by other HATs. Because many other transcription factors such as p53 are subject to the regulatory mechanisms of TIP60 and p300 (Ito et al., 2001; Tang et al., 2006), the acetylation of these factors may also be regulated by the same cooperative interplay of TIP60 and p300 discussed herein. This cooperative and complex interplay may represent defining features of a common regulatory mode of action of distinct HATs and their shared substrates.

EXPERIMENTAL PROCEDURES

Mice

p300 conditional knockout mice were kindly provided by Dr. Paul Brindle (St. Jude Children's Hospital, Memphis). Cre-recombinase-mediated excision was designed to remove exons 3–11, which comprise 71% of Tip60 exon structure, including the chromo-finger, Zinc finger, and HAT domains, by recombineering wherein LoxP sites were inserted into introns 2 and 11. Correctly targeted embryonic stem cells were injected into C57/Bl6 blastocysts, which, after implantation, transmitted the targeted allele via germline. Following verification of targeting via Southern blotting and removal of the neomycin-resistance gene, mice were bred to the genotypes used in these experiments (J.W.L., A. Horst, and J.B. Fisher, unpublished data). $Foxp3^{YFP\text{-}Cre}$ mice were obtained from Jackson Laboratory. All animals were housed and bred in a specific pathogen-free animal facility of the University of Pennsylvania. All the experiments were performed following national, state, and institutional guidelines. Animal protocols were approved by the University of Pennsylvania Animal Care and Use Committee.

Plasmids and Antibodies

The following antibodies were used in our studies: anti-Flag M2-Peroxidase (Sigma-Aldrich); anti-HA-Peroxidase (3F10; Roche); anti-ubiquitin-Peroxidase (sc-8017; Santa Cruz Biotechnology); and anti-acetyl-lysine (ICP0381; ImmuneChem). Plasmids expressing the WT or HAT-deficient TIP60 were constructed as previously described by Li et al. (2007). p300 was cloned from pCDNA3.1-p300 (kindly provided by Warner Greene; Addgene plasmid 23252) to pFLAG, resulting in pFLAG-p300. pFLAG-TIP60 K274R, K274Q, and pFLAG-p300 F1504A were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and verified by sequencing.

Cell Culture and Transfection

293T cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (1% penicillin/streptomycin; Invitrogen) at 37°C in a humidified incubator with 5% CO_2 (v/v). Cells were grown to 80% confluency, and transient transfection was carried out using a mixture of 6 μg DNA and 18 μl FuGENE 6 (Roche) according to manufacturer's instructions. Twenty-four hours after transfection, cells were washed twice with PBS, and cell lysates were then prepared for western blot analysis.

Immunoprecipitation

Cells were lysed in modified RIPA buffer (20 mM Tris-Cl [pH 7.5], 2 mM EDTA, 420 mM NaCl, and 1% NP40). After centrifugation, the soluble fractions were collected and incubated with anti-HA or anti-FLAG agarose (Sigma-Aldrich) overnight at 4°C. The precipitates were then washed three times with modified RIPA buffer and boiled for 5 min in SDS loading buffer. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose membrane (Millipore), and probed with antibodies as indicated. Immunocomplexes were detected using Immobilon Western Chemiluminescent horseradish peroxidase (HRP) Substrate (Millipore).

Flow Cytometry

Spleen, axillary and inguinal lymph node, mesenteric lymph node, and thymus of 18- to 21-day-old male mice were collected for single-cell suspension. Cells were stained with anti-CD4-percp and CD8-AF700 (eBioscience) and subjected to flow cytometry with fluorescence-activated cell sorting (FACS) LSR (BD Biosciences). FACS data were analyzed with FlowJo software (Tree Star).

Histology

Lung and liver tissues were fixed with 10% neutral-buffered formalin and embedded in paraffin. Sections were deparaffinized and stained with H&E by the Cell Imaging Core in the Abramson Cancer Research Institute.

CD4+CD25+ Suppression Assays

CD4+ T cells were enriched from splenocytes using MACS separation (Miltenyi Biotec), and CD4+CD25-CD45RBhigh Teff cells and CD4+CD5+CD45RBlow Treg cells were separated from CD4+ cells, respectively, by FACSAria II (BD Biosciences), yielding a purity of ~97% for both cells.

Luciferase Assay

Luciferase assays were performed as previously described by Li et al. (2007). Cells were transfected in a 12-well plate with pG5-luc, MSV-β-gal, pBIND-FOXP3, pFLAG-TIP60, and pFLAG-p300 as indicated. Twenty-four hours after transfection, cells were washed twice with PBS and lysed in 100 μl passive lysis buffer for 15 min. Luciferase and β-gal activities were then determined separately using the luciferase assay system and the galactosidase enzyme assay system, respectively (Promega).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.021.

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