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CITATION:
Okamura, Kazumi ...[et al]. Survival of mature T cells depends on signaling through HOIP.. Scientific reports 2016, 6: 36135.

ISSUE DATE:
2016-10-27

URL:
http://hdl.handle.net/2433/217250

RIGHT:
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Survival of mature T cells depends on signaling through HOIP

Kazumi Okamura1,2, Akiko Kitamura1, Yoshiteru Sasaki1, Doo Hyun Chung1, Shoji Kagami2, Kazuhiro Iwai2 & Koji Yasutomo1,4

T cell development in the thymus is controlled by a multistep process. The NF-κB pathway regulates T cell development as well as T cell activation at multiple differentiation stages. The linear ubiquitin chain assembly complex (LUBAC) is composed of Sharpin, HOIL-1L and HOIP, and it is crucial for regulating the NF-κB and cell death pathways. However, little is known about the roles of LUBAC in T-cell development and activation. Here, we show that in T-HOIPΔlinear mice lacking the ubiquitin ligase activity of LUBAC, thymic CD4+ or CD8+ T cell numbers were markedly reduced with severe defects in NKT cell development. HOIPΔlinear CD4+ T cells failed to phosphorylate IkBa and JNK through T cell receptor-mediated stimulation. Mature CD4+ and CD8+ T cells in T-HOIPΔlinear mice underwent apoptosis more rapidly than control T cells, and it was accompanied by lower CD127 expression on CD4+CD24low and CD8+CD24low T cells in the thymus. The enforced expression of CD127 in T-HOIPΔlinear thymocytes rescued the development of mature CD8+ T cells. Collectively, our results showed that LUBAC ligase activity is key for the survival of mature T cells, and suggest multiple roles of the NF-κB and cell death pathways in activating or maintaining T cell-mediated adaptive immune responses.

T cells express the T cell receptor (TCR) that recognizes a peptide presented by the MHC. T cells subsequently differentiate toward various effector cells that are required for combating microorganisms or tumor cells1-4. Importantly, excessive activation of effector T cells can lead to various diseases including autoimmune disorders5. CD4+CD8+ cells in the thymus receive TCR signals and the quantity or the quality of TCR signaling dictates the differentiation to mature CD4+ or CD8+ T cells6-8. Th-POK and RUNX3 are crucial transcription factors modulating the lineage differentiation to CD4+ or CD8+ T cells, respectively9-12. The relationship between TCR signaling and transcriptional regulation remains unclear. In the thymus, the differentiation of T cells beyond the CD4+CD8+ cell stage requires persistent TCR signaling13,14. Moreover, IL-7 receptor signaling is crucial for the final maturation or survival of CD4+ and CD8+ T cells in the thymus15,16.

The NF-κB family includes five related proteins, c-Rel, p65, RelB, p50 and p52. Those proteins form homodimers and heterodimers in specific combinations together with a regulatory protein, the inhibitor IκB.β family includes five related proteins, c-Rel, p65, RelB, p50 and p52. Those proteins form homodimers and heterodimers in specific combinations together with a regulatory protein, the inhibitor IκB. β family members in thymocyte differentiation and maturation following TCR receptor-mediated stimulation. Mature CD4+ and CD8+ T cells in T-HOIPΔlinear mice underwent apoptosis more rapidly than control T cells, and it was accompanied by lower CD127 expression on CD4+CD24low and CD8+CD24low T cells in the thymus. The enforced expression of CD127 in T-HOIPΔlinear thymocytes rescued the development of mature CD8+ T cells. Collectively, our results showed that LUBAC ligase activity is key for the survival of mature T cells, and suggest multiple roles of the NF-κB and cell death pathways in activating or maintaining T cell-mediated adaptive immune responses.

1Department of Immunology & Parasitology, Graduate School of Medicine, Tokushima University, Tokushima, Japan. 2Department of Pediatrics, Graduate School of Medicine, Tokushima University, Tokushima, Japan. 3Department of Molecular and Cellular Physiology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. 4Department of Pathology, Seoul National University College of Medicine, Seoul, Korea. 5Core Research for Evolutional Science and Technology, Japan Agency for Medical Research and Development, Tokyo, Japan. Correspondence and requests for materials should be addressed to K.Y. (email: yasutomo@tokushima-u.ac.jp)
lymphangiectasia. However, the precise role of HOIP or LUBAC ligase activity in T cell development is poorly understood.

Here, we demonstrated that T cell-specific T-HOIPΔlinear mice showed impairments of mature T cell development and proliferative responses. Those data highlighted the HOIP-mediated NF-κB pathway as a crucial pathway in the regulation of T cell development. Furthermore, our data indicated that deficiency of LUBAC ligase activity disturbed the development of mature T cells and their function, suggesting the important role of LUBAC for T cell-mediated adaptive immune responses.

Results

The deficiency of Rnf-31 ligase activity in T cells impaired the development of mature T cells in the thymus. To evaluate the involvement of HOIP in T cell development, we established Rnf31Δlinear/Δlinear mice with a CD4-Cre transgene (T-HOIPΔlinear mice). The frequency of TCR3+ cells in the thymus was reduced in T-HOIPΔlinear mice and the relative and absolute numbers of CD4+CD8− and CD4−CD8+ cells were markedly reduced in T-HOIPΔlinear mice whereas CD4+CD8+ cells were not depressed (Fig. 1a,b). The effect was much stronger in CD4+CD8− cells than CD4−CD8+ cells. The frequency of TCR3+ cells in T-HOIPΔlinear mice was equivalent to that of Rnf31Δ/+ mice with CD4-Cre transgene (HOIPΔ/+ mice) (Fig. 1a). Mature CD4+CD8− cells and CD4−CD8+ T cells in the thymus downregulate CD24 and CD69 during the final maturation steps. T-HOIPΔlinear mice had relatively higher frequencies of CD24-positive and CD69-positive cells in both CD4+CD8− TCRβ+ and CD4−CD8+ TCRβ+ fractions than did HOIPΔ/+ mice (Fig. 1c). These results suggested that HOIP-mediated ligase activity was required for final maturation or survival of mature CD4+CD8− and CD4−CD8+ T cells in the thymus.
T-HOIPΔlinear mice had lower numbers of mature T cells in the spleen and lymph nodes. We next assessed the T cell numbers and phenotypes in the spleen and lymph nodes of T-HOIPΔlinear mice. The relative frequencies of TCRβ+ to TCRγ+ cells or TCRβ+ cells to B220+ cells was markedly reduced in the spleen and lymph nodes of T-HOIPΔlinear mice (Fig. 2a). The total cell numbers of TCRβ+, CD4+ and CD8+ T cells in the spleen and lymph nodes in T-HOIPΔlinear mice were also much less than in control mice (Fig. 2a). The relative frequency of CD8+ cells to CD4+ cells was reduced in HOIPΔ−/− mice in the spleen (Fig. 2a). T-HOIPΔlinear mice possessed higher numbers of CD44hiCD62LloCD4+ and CD44hiCD62LloCD8+ T cells compared with control mice (Fig. 2b), suggesting that mature T cells from T-HOIPΔlinear mice had undergone activation after being exported from the thymus. The relative frequency of CD4+Foxp3+ regulatory T cells in CD4+ T cells was not affected in the lymph nodes of T-HOIPΔlinear mice (Fig. 2c), whereas the frequency of CD4+CD1d tetramer+ NKT cells was reduced in the thymus and liver of T-HOIPΔlinear mice (Fig. 2d). Taken together, those data demonstrated that HOIP-deficiency in T cells markedly impaired the differentiation or survival of both mature CD4+ and CD8+ T cells with striking defects in the development of NKT cells.

CD4+ T cells in T-HOIPΔlinear mice proliferated poorly after TCR ligation. We evaluated the in vitro proliferative ability of CFSE-labeled CD4+ T cells from T-HOIPΔlinear mice when stimulated by anti-CD3 mAb...
exposure. Those CD4⁺ T cells showed less CFSE dilution than did control cells, indicating relatively slower proliferative activity (Fig. 3a). The poorer proliferative activity of CD4⁺ T cells from T-HOIPΔlinear mice was not rescued by the addition of IL-2 to the culture medium (Fig. 3a). To assess the role of HOIP in the functional differentiation of CD4⁺ T cells, we immunized T-HOIPΔlinear mice and HOIP+/+ mice with OVA protein and evaluated anti-OVA specific immunoglobulin production and levels of serum cytokines 14 days after immunization.

The CD4⁺ T cells from OVA-immunized T-HOIPΔlinear mice produced lower amounts of IFN-γ than those from HOIP+/+ mice (Fig. 3b) and failed to produce anti-OVA-specific IgG, IgG1 and IgG2c (Fig. 3c).

CD4⁺ T cells in T-HOIPΔlinear mice failed to phosphorylate IκBα. We assessed the role of HOIP ligase in the activation of NF-κB in mature T cells. Thus, CD4⁺ T cells from T-HOIPΔlinear mice and HOIP+/+ mice were stimulated by anti-CD3 mAb and phosphorylation of IκBα was evaluated. Anti-CD3 mAb treatment of CD4⁺ T cells from T-HOIPΔlinear mice induced less phosphorylation of IκBα than observed in cells from control mice (Fig. 4a). We then analyzed nuclear translocation of NF-κB (p65) after anti-CD3 mAb-stimulation of CD4⁺ T cells from T-HOIPΔlinear mice or HOIP+/+ mice. Little nuclear translocation of p65 was found in CD4⁺ T cells from T-HOIPΔlinear mice compared with efficient translocation of p65 into the nucleus in control T cells (Fig. 4b). The deficiency of HOIP ligase activity in B cells disturbs CD40 but not B cell receptor-mediated JNK activation. Thus, we analyzed TCR-mediated JNK phosphorylation in T-HOIPΔlinear mice T cells. Stimulation with anti-CD3 mAb induced less phosphorylation of JNK in HOIP ligase-deficient T cells compared with control cells (Fig. 4c). Those data demonstrated that the deficiency of HOIP ligase activity disturbed activation of not only the canonical NF-κB pathway but also the JNK pathway.
T cells from HOIP-deficient mice lost viability. We sought to assess whether the loss of mature T cells in T-HOIPΔlinear mice was attributable to increased cell death. Thus, mature CD4+ or CD8+ T cells from the thymus and spleen were stained with Annexin V and 7AAD. Larger percentages of CD8+ T cells in the thymus and CD4+ or CD8+ T cells in the spleen of T-HOIPΔlinear mice were positive for Annexin V than in control cells (Fig. 5a). Those data suggested that the deficiency of HOIP ligase activity increased the frequency of cell death in mature T cells, especially in early developmental stages of single positive cells in the thymus.

To confirm that CD4+ or CD8+ T cells from T-HOIPΔlinear mice did not retain viability, we compared cell survival of CD4+ T cells from T-HOIPΔlinear mice and HOIP+/+ mice. When CD4+ T cells from T-HOIPΔlinear mice (CD45.2) and control (CD45.1) mice were cultured in vitro without any stimulation or after stimulation with anti-CD3 mAb, T cells from T-HOIPΔlinear mice died more rapidly than those from HOIP+/+ mice (Fig. 5b). To determine if the impaired T cell survival also occurred in vivo, CD4+ T cells (CD45.2) from T-HOIPΔlinear mice or HOIP+/+ mice were transferred into recipient C57BL/6 (CD45.1) mice. The number of CD4+ cells from T-HOIPΔlinear mice was much lower than control cells 3 days after transfer into inguinal lymph nodes, (Fig. 5c). Those results suggested that CD4+ T cells from HOIP−/− mice were prone to die compared with control CD4+ T cells.

CD127 expression was lower in T cells from T-HOIPΔlinear mice. In order to determine the molecular mechanisms for impaired development of T cells from T-HOIPΔlinear mice, we tested the expression of cytokine receptors on T cells. The expression levels of common γ-chain (CD132) were comparable between CD4+ and CD8+ T cells in T-HOIPΔlinear mice and HOIP+/+ mice (Fig. 6a). However, the expression levels of IL-2Rs (CD25) and IL-2Rβ (CD122) were higher in splenic CD8+ T cells from T-HOIPΔlinear mice. Moreover, the expression levels of IL-7Rs (CD127) were relatively high in CD4+ and CD8+ splenic T cells from T-HOIPΔlinear mice (Fig. 6a). In contrast, the expression of CD127 was lower in thymic CD4+ and CD8+ T cells from T-HOIPΔlinear mice (Fig. 6b).

Il7r (CD127) was reported to be a target gene for NF-κB signaling25. As IL-7 is required for CD8+ T cell survival, we examined if impaired development of T cells from T-HOIPΔlinear mice was attributable to low

Figure 4. Impaired NF-κB and JNK activation in T-HOIPΔlinear T cells. (a) Isolated CD4+ T cells from HOIP+/+ (blue) or T-HOIPΔlinear (red) mice were stimulated with anti-CD3 mAb followed by anti-hamster IgG for the indicated time. The expression of phospho-IκBα was evaluated by flow cytometry. As the negative control, staining with isotype control IgG was used (filled gray). (b) Isolated CD4+ T cells from HOIP+/+ or T-HOIPΔlinear mice were stimulated with anti-CD3 mAb followed by anti-hamster IgG for the indicated time. The expression of p65 (red) in CD4+ T cells ten min after stimulation were evaluated by confocal microscopy. The nucleus was stained with DAPI (blue). (c) Isolated CD4+ T cells from HOIP+/+ (blue) or T-HOIPΔlinear (red) mice were stimulated with anti-CD3 mAb followed by anti-hamster IgG for the indicated time. The expression levels of phospho-JNK were evaluated by flow cytometry. As the negative control, staining with isotype control IgG was used (filled gray). The data in these figures are representative of four independent experiments.
CD127 expression. CD127-encoding retrovirus was infected in fetal thymocytes from T-HOIP$\Delta$linear mice. The GFP-expressing thymocytes were cultured in fetal thymus for 7 days and the development of mature T cells was examined. The overexpression of CD127 increased the frequency of mature CD8$^+$ TCR$\beta^+$ but not CD4$^+$ TCR$\beta^+$ T cells (Fig. 6c). Those data indicated that impaired CD8$^+$ T cell survival in T-HOIP$\Delta$linear mice is, at least partly, attributable to low CD127 expression.

**Discussion**

LUBAC-mediated poly-linear ubiquitination is a crucial event for activating the NF-κB pathway$^{21,20}$. However, the roles of LUBAC-mediated NF-κB regulation in T cell activation or in development have been unresolved. In this paper, we show that among the LUBAC components, HOIP ligase activity is required for the development of mature T cells and is crucial for CD4$^+$ T cell proliferation. T-HOIP$\Delta$linear mice T cells failed to upregulate CD127, which was attributable to the impaired survival of thymic CD8$^+$ T cells but not CD4$^+$ T cells in T-HOIP$\Delta$linear mice. These findings demonstrate the crucial contribution of HOIP-mediated linear ubiquitination of NEMO to T cell development. They support a model in which CD4$^+$ and CD8$^+$ T cells have distinct molecular requirements for NF-κB-mediated molecules downstream.

T cell development in the thymus is controlled by a multistep process utilizing the TCR, costimulatory molecules and cytokine signals, each of which is required during specific stages of development. Given that the TCR and cytokines signaling are crucial for T cell development, with NF-κB downstream for various receptors in conventional T cells, HOIP could control thymic T cell differentiation at multiple points. Our data demonstrated that mature CD4$^+$ or CD8$^+$ T cells were markedly diminished with reduced expression of CD127 in T-HOIP$\Delta$linear mice, a deficit that was rescued by overexpressing CD127 on CD8$^+$ T cells, at least in an in vitro culture system. IL-7 functions in the survival and development of conventional CD4$^+$ and CD8$^+$ T cells, as evidenced by a markedly reduced number of mature CD4$^+$ and CD8$^+$ T cells in CD127-deficient mice$^{15}$. Therefore, the impaired survival of CD8$^+$ T cells in T-HOIP$\Delta$linear mice could be, at least partially, attributable to the reduced expression of CD127. In contrast, the development of CD4$^+$ T cells could not be rescued by overexpressing CD127, suggesting...
that the dysregulation of other target molecules downstream from HOIP is responsible for the impaired survival of CD4+ T cells. Those data suggest a model in which CD4 and CD8 T cells require distinct regulation of target molecules downstream of HOIP for their survival in the thymus.

HOIP complexes with HOIL-1L and SHARPIN.26,22 Mutations in the murine Sharpin gene cause spontaneous chronic proliferative dermatitis (cpdm) that develops into psoriasis-like proliferative skin lesions, splenomegaly, absence of Peyer's patches and low levels of serum immunoglobulin.27 A recent study reported that patients with a loss-of-function mutation in HOIL-1L suffered from chronic autoinflammation, invasive bacterial infections and muscular amylopectinosis.28 Furthermore, an inherited mutation in HOIP causes multi-organ autoinflammation, combined immunodeficiency, subclinical amylopectinosis, and systemic lymphangiectasia.23 These findings suggest a distinct requirement for each LUBAC subunit to control downstream pathways. In contrast to the autoinflammatory phenotypes associated with HOIP- or HOIL-1L-deficiency in humans, the present study revealed that a deficiency in HOIP ligase activity impaired NF-κB activation leading to the impairment of both CD4+ and CD8+ T cell development without any inflammatory responses. As LUBAC ligase activity was deleted only in T cells in our mouse study, the loss of function of non-T cells might be involved in the development of inflammatory responses.

The CBM (CARMA1–Bcl10–Malt1) TCR adaptor complex regulates TCR-dependent NF-κB activation.29–31 Despite the important roles of CARMA1 in NEMO activation, CARMA1-deficient mice have normal T-cell development and normal peripheral T-cell numbers and ratios.2,33 However, they do have a defect in the development of intrathymic CD4+CD25+ regulatory T cells. In contrast, T cell-specific, NEMO-deficient mice are devoid of mature CD4+ and CD8+ T cells in the thymus, a finding that is similar to T cell-specific, HOIP ligase activity deficient mice. Furthermore, HOI-ligase activity deficient CD4+ T cells have a defect in TCR-mediated proliferation and NF-κB activation. Those results suggest that the engagement of TCR activates NEMO by utilizing CARMA1-dependent or -independent pathways, and that LUBAC-mediated linear ubiquitination of NEMO through engagement of TCR is essential for the survival of mature T cells. In addition, a recent paper revealed that the dysregulation of other target molecules downstream from HOIP is responsible for the impaired survival of CD4+ T cells. Those data suggest a model in which CD4 and CD8 T cells require distinct regulation of target molecules downstream of HOIP for their survival in the thymus.

Figure 6. Defective IL-7Rα in thymocytes of T-HOIPΔlinear mice. (a) Spleen cells from T-HOIPΔlinear (red) or HOIPΔ+/+ (black) mice were stained with anti-CD4, anti-CD8α, anti-CD25, anti-CD122, anti-CD127 and anti-CD132 antibodies. The expression of CD25, CD122, CD127 and CD132 in CD4+CD8− (CD4SP) or CD4−CD8+ (CD8SP) was evaluated by flow cytometry. The negative control cells were stained with isotype controls (filled gray). (b) Thymocytes from T-HOIPΔlinear or HOIPΔ+/+ mice were stained with anti-CD4, anti-CD8α, anti-CD24 and anti-CD127 antibodies. The expression of CD127 by CD4+CD8−CD24hi or CD4+CD8−CD24lo or CD4−CD8+CD24hi or CD4−CD8+CD24lo cells was evaluated by flow cytometry. As the negative control, cells were stained with isotype controls (filled gray). The number indicates the mean fluorescence intensity (MFI) of each population in the viable population. (c) Fetal thymocytes (day 15 fetal age) were infected with control retrovirus (EV) or retrovirus containing the CD127 gene (Il7r) and cultured in dGu-treated fetal thymus for 7 days. Thymocytes were stained with anti-CD4 and anti-CD8α antibodies and the expression gated on GFP+ cells was evaluated by flow cytometry. The number indicates the percentage of each population in the viable population. The data in these figures are representative of four independent experiments.
that LUBAC integrates the CBM complex and that NF-κB reporter activity is stimulated following antigen receptor ligation independent of its catalytic activity35. However as this study was performed by evaluating NF-κB reporter activity in Jurkat cells that had been transfected with siRNA against HOIP and siRNA resistant ligase activity-inactive HOIP, the effect of the residual activity of endogenous HOIP might not be negligible. In future studies, it will be necessary to evaluate which domains of HOIP are crucial for binding with the CBM complex.

In this report, we found that canonical NF-κB signaling through linear ubiquitination by LUBAC was an essential molecular pathway that regulated CD4+ and CD8+ T cell development. Our data highlight a previously unknown molecular link between LUBAC and mature CD4+ and CD8+ T cell survival. Those data also suggest new approaches for inhibiting HOIP ligase activity and thereby suppressing T-cell-mediated immune responses.

Methods

Mice. Six- to 8-week-old C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Rnf31+/- mice were previously described24. C57BL/6 mice (CD45.1) and CD4–Cre transgenic mice were purchased from Jackson Laboratory (MA, USA). All animal experiments were approved by an animal research ethical committee of Tokushima University and were performed according to its guidelines.

Flow cytometric analysis. The livers were homogenized and resuspended in gradient buffer that contained 2.5% FBS plus 10 mL Percoll (GE Healthcare) and 2 mL Alsever’s solution (Sigma-Aldrich). Thymocytes, spleenocytes or lymph nodes cells were filtered through a 100 μm mesh. Fluorochrome-conjugated monoclonal antibodies specific for mouse CD8ε (53–6.7), CD44 (IM7) and Fcγ3 (3G3) were purchased from Tombo Biosciences (San Diego, CA, USA). Antibodies specific for CD4 (GK1.5), CD122 (TM β1), B220 (RA3-6B2) and TCRγ (GL3) were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies specific for CD24 (M1/69), CD25 (3C7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD127 (A7R34), CD132 (TU-Gm2), NK1.1 (PK136), and TCRβ (H57-597) were bought from BioLegend (San Diego, CA, USA). The CD1d tetramer was provided from the NIH tetramer facility. Antibodies specific for phospho-IκB (G9) were purchased from Cell Signaling Technology (Danvers, MA, USA). All samples were resuspended in PBS staining buffer containing 2% FBS and 0.01% NaN₃, and pre-incubated for 15 min at 4°C with 2.4G2 supernatant to block Fc receptor, then washed and stained with specific mAbs for 20 min at 4°C. For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin-containing buffer. Data were collected on a FACS Canto II (BD Biosciences) and analyzed using FACS Diva (BD Biosciences) or FlowJo (Tree Star, OR, USA) software.

T cell proliferation analysis. CD4+ T cells were isolated with anti-CD4 microbeads (Miltenyi Biotech, Germany) and labeled with CFSE (10 μg/mL). CD4+ T cells (3 × 10⁵/well; 24-well plates) were stimulated with plate-coated anti-CD3 mAb (145-2C11) (1 μg/mL) (Tombo Biosciences) in the absence or presence of mouse recombinant IL-2 (10 U/mL) (Miltenyi Biotec) for 3 days.

OVA immunization. Mice were immunized with OVA protein (50 μg) emulsified in CFA (Sigma, Saint Louis, MO, USA) and the titers of OVA-specific antibodies (IgG, IgG1 and IgG2c) were measured by ELISA using HRP-conjugated anti-mouse IgG, IgG1 or IgG2c (Southern Biotech, Alabama, USA) as the secondary antibodies.

T cell survival. Purified CD4+ T cells (4 × 10⁶) from C57BL/6 (CD45.1) and HOIP+/− (CD45.2) mice were cultured without any stimulation or stimulated with plate-coated anti-CD3 mAb (1 μg/mL). After culturing cells, cell number was counted, cells were stained with anti-CD4, CD45.1 and CD45.2 antibodies and the number of cells in each population was calculated.

ELISA. ELISA for IFN-γ was performed using an ELISA kit from ebioscience.

Confocal laser-scanning microscopy analyses. T cells were isolated with a pan-T cell isolation kit (Miltenyi Biotec) and stimulated for 10 min at 37°C with anti-CD3 mAb followed by anti-hamster IgG. Cells were then seeded on poly-L-lysine-hydrobromide-coated cover glass, fixed with 4% paraformaldehyde and permeabilized with acetone. Staining with anti-p65 mAb (1 μg/mL) (Santa Cruz Biotech) was followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen). The nucleus was stained with DAPI. The observations were performed using an FV10i confocal microscope (OLYMPUS, Japan). Several cells were analyzed for each labeling condition, and representative results are presented.

Fetal thymic organ culture. Fetal thymus (fetal age, day 15) from C57BL/6 mice was cultured in the presence of deoxyguanosine (1.35 mM) (Sigma) on Transwell plates for 7 days. Fetal thymocytes (fetal age, day 15) from HOIP+/− or HOIP−/− mice were isolated and infected with control retrovirus or Il7r-encoding-retrovirus as previously reported36. The pKE004 retrovirus vector37 that encodes IRES-GFP and Il7r was transduced into Plat-E cells to generate retrovirus. The infected thymocytes were cultured for one day in the presence of IL-7 (5 ng/mL) (ebioscience). Thymocytes were hanging-drop cultured with deoxyguanosine-treated thymus using a Terasaki plate for one day. Then the thymus was cultured on a Transwell plate for 7 days.

Statistical analysis. For all experiments, the significance of differences between groups was calculated using the Mann-Whitney U test for unpaired data. Differences were considered significant when p < 0.05.
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Acknowledgements

We thank Mrs. C. Kinouchi and C. Miyamoto for technical and editorial assistance. This research was supported by AMED-CREST.
Author Contributions
K.O. performed all experiments. A.K., Y.S., K.I., D.H.C. and S.K. analyzed the data. K.Y. supervised the studies and K.O. and K.Y. wrote the paper. All authors reviewed the manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Okamura, K. et al. Survival of mature T cells depends on signaling through HOIP. Sci. Rep. 6, 36135; doi: 10.1038/srep36135 (2016).

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