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RESEARCH ARTICLE

Retinoblastoma 1 protects T cell maturation from premature apoptosis by inhibiting E2F1

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ABSTRACT

T lymphocytes are key cellular components of an acquired immune system and play essential roles in cell-mediated immunity. T cell development occurs in the thymus where 95% of immature thymocytes are eliminated via apoptosis. It is known that mutation of *Zeb1*, one of the retinoblastoma 1 (Rb1) target genes, results in a decrease in the number of immature T cells in mice. E2F1, an RB1-interacting protein, has been shown to regulate mature T cell development by interfering with thymocyte apoptosis. However, whether Rb1 regulates thymocyte development *in vivo* still needs to be further investigated. Here, we use a zebrafish model to investigate the role of Rb1 in T cell development. We show that Rb1-deficient fish exhibit a significant reduction in T cell number during early development that it is attributed to the accelerated apoptosis of immature T cells in a caspase-dependent manner. We further show that E2F1 overexpression could mimic the reduced T lymphocytes phenotype of Rb1 mutants, and E2F1 knockdown could rescue the phenotype in Rb1-deficient mutants. Collectively, our data indicate that the Rb1-E2F1-caspase axis is crucial for protecting immature T cells from apoptosis during early T lymphocyte maturation.

KEY WORDS: Rb1, E2F1, Apoptosis, T lymphocyte, Zebrafish

INTRODUCTION

T lymphocytes are key players in cell-mediated immunity. T lymphocyte progenitors are derived from hematopoietic stem cells (HSCs) and then developed for maturation in the thymus (Kondo et al., 1997). Lymphocytes must be able to produce diverse and specific antigen receptors to fight against invading pathogens. These receptors are coded by sequences with different variable (V) regions. However, this stochastic process is prone to generate antigen receptors that are nonfunctional or self-reactive. To avoid autoimmunity, self-reactive lymphocytes must be eliminated (Kruisbeek and Amsen, 1996).

Apoptosis is one mechanism by which immature self-reactive T lymphocytes are eliminated during maturation (Sohn et al., 2007). The transcription factor E2F1, a well-known retinoblastoma susceptibility (RB1)-interacting protein, influences apoptosis during thymic negative selection (Zhu et al., 1999); By using *E2f1*$^{-/-}$ mice, Field et al. determined that loss of E2F resulted in a mature stage T cells (CD4^{+} or CD8^{+} single-positive) increased, presumably owing to decreased apoptosis during negative selection (Field et al., 1996). After that, by using mouse embryo fibroblasts, more-recent studies have shown that RB1/E2F1 is able to bind to the *Ets1* and *Zeb1* promoters to repress their expression (Dean et al., 2015; Liu et al., 2007). In addition, *Zeb1*$^{-/-}$ mice show a decrease number in multiple stage of thymocytes, including intrathymic *Kit*$^{+}$ T precursor cells in T early stage (Dean et al., 2015; Higashi et al., 1997). In a clinical setting, somatic mutation of *RB1* is the most common genomic abnormality in ∼21% of chronic lymphocytic leukaemia (CLL) cases (Ouillette et al., 2011; Puiggros et al., 2014). Thus, previous studies have suggested that Rb1 may be involved in thymocyte development, but there is no direct *in vivo* evidence to prove a role for Rb1 in the process. Whether Rb1 has effects on thymocytes and whether it is in an E2F1-dependent manner are still unclear. And the stage of the affected thymocytes (immature or mature) also needs to be clarified. Furthermore, the mechanism underlying the apoptosis pathway remains to be uncovered.

In this study, we generated a Rb1 loss-of-function zebrafish mutant (*rb1*$^{smu8/smu8}$) using TALENs (Dee et al., 2016; Langenau and Zon, 2005; Trede et al., 2004). By using the *rb1*$^{smu8/smu8}$ zebrafish model, we show that Rb1 is necessary for cell apoptosis during early T lymphocyte maturation. We found that T cell numbers in the Rb1-deficient mutant were inadequate, whereas development of other hematopoietic cells was unaffected. The reduction of T cells in *rb1*$^{smu8/smu8}$ mutants resulted from premature cell apoptosis mediated by elevated caspase 3 activity. We further demonstrated that downregulation of E2F1 could rescue the inappropriate apoptosis in *rb1*$^{smu8/smu8}$ mutants. Our findings suggest that Rb1 can inhibit E2F1 from triggering the caspase cascade during early T lymphocyte maturation.

RESULTS

TALEN mediates *rb1* gene knockout in zebrafish

To investigate whether Rb1 has roles during T cell maturation, we used TALEN targeting to isolate germline mutations in the zebrafish retinoblastoma susceptibility gene (*rb1*). Zebrafish embryos were injected with TALEN mRNA targeting *rb1* exon 2 with high efficiency, obtaining up to 80% allelic loss in injected F0 embryos (Fig. 1A). We screened one F1 adult for germline transmission *rb1* alleles using an 8 bp (referred as *rb1*$^{smu8/smu8}$) frameshift deletion, in which a truncated Rb1 protein lacking all functional domains should be produced (Fig. 1A). As confirmed by western blotting analysis, Rb1 protein was absent in the mutants (Fig. 1B). F2 homozygous larvae failed to develop swim bladders and died at ∼15 dpf (Fig. 1C), similar to the previously reported Rb1 mutant *space cadet* (Gyda et al., 2012).

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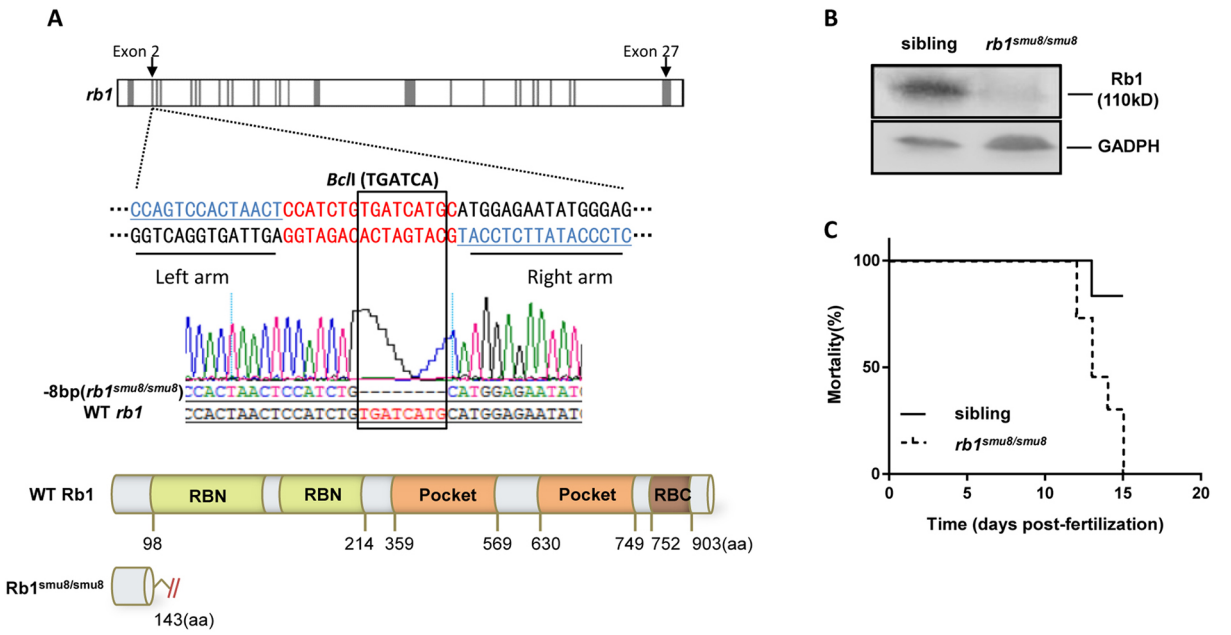

Fig. 1. TALEN mediates *rb1* gene knockout in zebrafish. (A) The zebrafish *rb1* gene structure (top). Exons are indicated by grey boxes. Location and sequence of the TALEN target site for the *rb1* gene is magnified. Sequence flanking the TALEN target site in $rb1^{smu8/smu8}$ F_2 embryos. The deletion of eight nucleotides is shown in the black box. *Bcl*I, genotyping enzyme. Rb1 protein structures in wild type and $rb1^{smu8/smu8}$ mutant (bottom). Red slashes indicate the premature stop of the Rb1 protein. (B) Examination of Rb1 expression in the whole fish body by western blot at 5 dpf. (C) Average mortality curve (percentage) of the siblings and *rb1*-deficient embryos (n=20).

T lymphocyte maturation is impaired in the absence of Rb1

To investigate the effect of Rb1 loss on T lymphocyte development, we examined the expression of T-cell markers in $rb1^{smu8/smu8}$ mutant using whole mount *in situ* hybridization. We examined the expression of *ikaros* (*ikzf1*) the marker for both immature and mature lymphocytes (Willett et al., 2001), and found that its expression was slightly reduced in the thymus of the mutants at 5 dpf (Fig. 2A), suggesting that thymic lymphocytes are impaired. Levels of the T-cell-specific tyrosine kinase gene (*lck*), which is expressed in thymic T cells in both immature and mature thymocytes (Langenau et al., 2004), were markedly decreased in the thymus of mutants compared with siblings (Fig. 2A). Furthermore, the expression of *rag1*, which encodes the recombinase responsible for recombination of the V(D)J and T- and B-cell antigen receptor genes (Wienholds et al., 2002), was also severely downregulated at 5 dpf in the mutants (Fig. 2A), suggesting that immature T cells are reduced in $rb1^{smu8/smu8}$ mutants. To validate this observation, by recording the number of lymphocytes in the thymus of *Tg(rag2:DsRed)* transgenic zebrafish from 3 dpf to 8 dpf, we found that the number of the $DsRed^{+}$ T cells was greatly decreased in mutants (Fig. 2B). Consistently, the qPCR analysis also showed that levels of *rag2* and *cd4* (*cd4-1*) were much lower in the mutant thymus (Fig. 2C), indicating the block of early T cell maturation in the $rb1^{smu8/smu8}$ mutant. In addition, levels of the thymic epithelial cell marker *foxn1* (Schorpp et al., 2002) and the HSC marker *myb* in the thymus were not altered in $rb1^{smu8/smu8}$ mutants (Fig. 2C-E), suggesting the T-cell deficiency is not caused by a HSC defect or a failure in thymus development. Except for T-cell markers, expression of other hematopoietic markers [*myb* for HSPCs (Zhang et al., 2011), *gata1* and *βe1* (*hbbe1*) for erythrocytes (Belele et al., 2009) and *pu.1* (*spi1b*), *mfap4* and *lyz* for myelocytes (Kitaguchi et al., 2009; Zakrzewska et al., 2010)] is unaltered in $rb1^{smu8/smu8}$ embryos (Figs S1A-D and S2A-F). Collectively, these data indicate that early T-cell development is disturbed in the absence of Rb1, whereas other hematopoietic lineages are not affected. To determine why *rb1* plays specific role in T cells, we further compared *rb1* expression in lymphocytes with that in other hematopoietic lineages. As expected, we found that *rb1* was highly expressed in $rag2{:}DsRed^{+}$ T cells, but expressed at lower levels in $lyz{:}DsRed^{+}$ granulocytes, $mpeg1{:}DsRed^{+}$ macrophages and $globin{:}DsRed^{+}$ erythrocytes (Fig. S2H), suggesting its specific function in the T-cell lineage.

Subsequently, we attempted to determine whether the impaired expression of lymphocytic markers in the thymus arose from the deficiency of Rb1 in T cells. The *rb1* expression construct driven by the *lck* or *rag2* promoter was injected into $rb1^{smu8/smu8}$ embryos to rescue the quantity of T cells. As shown by *rag1* whole-mount *in situ* hybridization or *rag2:DsRed* fluorescence, T cells in $rb1^{smu8/smu8}$ mutants were markedly rescued by *rb1* restoration (Fig. 2F and Fig. S3). These rescue experiment showed that delivery of *rb1* to T cells can rescue T cell loss in $rb1^{smu8/smu8}$ embryos effectively, suggesting a cell-autonomous role for Rb1 in early T-cell development. This result confirms that the defect in development of early T lymphocytes is caused by the absence of Rb1.

Rb1 deficiency results in T-cell apoptosis by increasing caspase 3 activity

Several possibilities could explain the reduction of early T lymphocytes, including reduced proliferation and increased cell death. When compared with siblings, the number of proliferating T cells in the thymus was not decreased in $rb1^{smu8/smu8}$ mutants, as indicated by the bromodeoxyuridine (BrdU)/*rag2:dsRed* incorporation assay (Fig. 3A). On the other hand, as indicated by the TUNEL assay, the number of apoptotic early T lymphocytes was significantly increased in $rb1^{smu8/smu8}$ embryos compared with siblings (Fig. 3B), demonstrating that the reduction in the number of early T lymphocytes in $rb1^{smu8/smu8}$ is attributed to increased apoptosis. We further examined the apoptotic T-cell numbers in *lck:rb1*-injected $rb1^{smu8/smu8}$ mutants to see whether the increased apoptosis could be rescued. Results show that the apoptotic T-cell numbers in $rb1^{smu8/smu8}$

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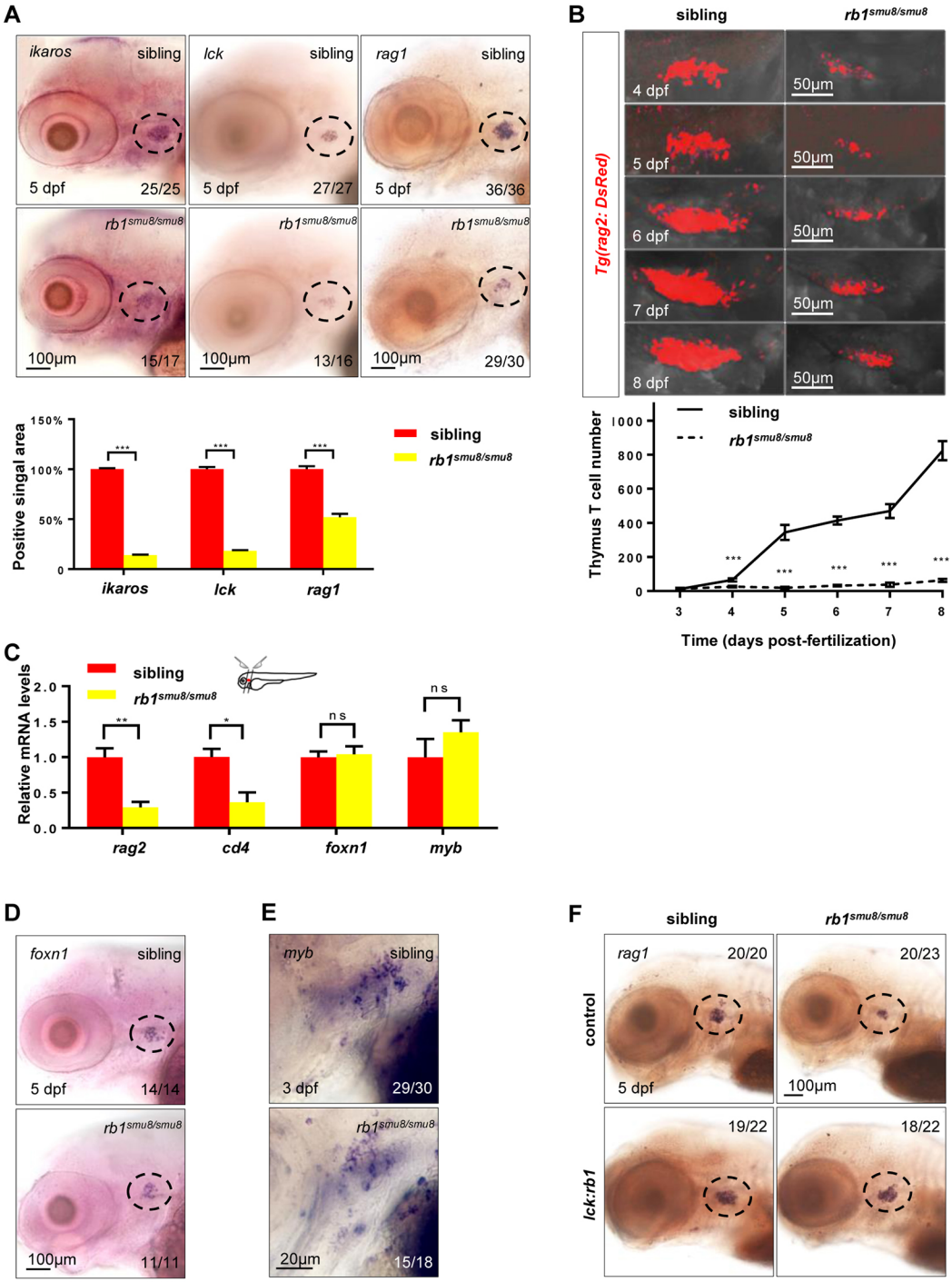

Fig. 2. T-cell maturation is impaired in *rb1*-deficient embryos. (A) Expression of *ikaros*, *lck* and *rag1* in the thymus (broken line) of siblings and *rb1* mutants at 5 dpf (upper panels). Embryos for whole-mount *in situ* hybridization were obtained from an incross of genotyped heterozygous *rb1* mutants. Scale bars: 100 µm. The positive signal areas were analysed using Image-Pro Plus (lower panel) (***P<0.001; mean±s.e.m.; n=16). (B) Confocal images of *rag2:DsRed* cells in the thymus of the siblings and *rb1*$^{smu8/smu8}$ mutants from 4 dpf to 8 dpf (upper panels). Scale bars: 50 µm. Quantification of T-cell number in sibling embryos and *rb1*$^{smu8/smu8}$ mutants (lower panel) (***P<0.001; mean±s.e.m.; n=10). (C) Schematic of the lateral view of a zebrafish indicating the region of the thymus excised for RNA extraction (red dot). qPCR analysis of *rag2*, *cd4*, *foxn1* and *myb* in the thymus of siblings and *rb1*-deficient embryos at 5 dpf (mean±s.e.m.; **P<0.01, *P<0.05, ns, not significant; n=30). (D) Whole-mount *in situ* hybridization of *foxn1* in the thymus (broken line) of siblings and *rb1*$^{smu8/smu8}$ mutants at 5 dpf. Scale bars: 100 µm. (E) Whole-mount *in situ* hybridization of *cmyb* in the thymus of the siblings and *rb1*$^{smu8/smu8}$ mutants at 3 dpf. Scale bars: 20 µm. (F) Whole-mount *in situ* hybridization of *rag1* in the thymus (broken line) at 5 dpf after injecting with control and pTol-*lck:rb1* plasmid. Scale bars: 100 µm.

mutants were markedly reduced by *rb1* restoration (Fig. 3C), indicating that the Rb1 loss-induced apoptosis is indeed the reason for T-cell loss. Taken together, these results reveal that Rb1 is involved in the early immature T lymphocyte development by inhibiting their apoptosis.

Previous studies in cancer cells have shown that lack of Rb1 promotes chromosome segregation errors, whereas loss of *p53* (*tp53*)allows tolerance and the continued proliferation of these unstable aneuploid cells (Manning et al., 2014). To examine

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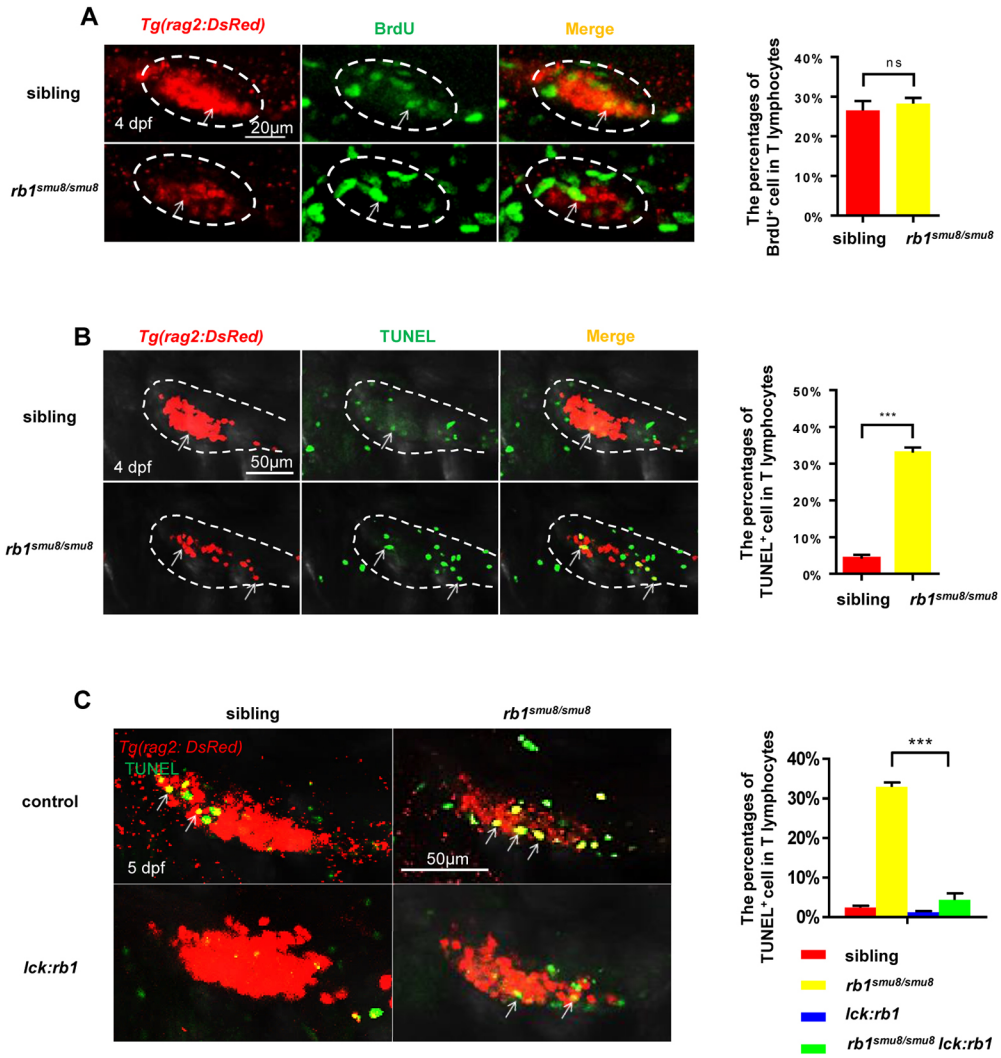

Fig. 3. Excessive apoptotic early T lymphocytes in the thymus of *rb1*$^{smu8/smu8}$ mutants. (A) Double staining of BrdU/*rag2-dsRed* show BrdU incorporation of T cells in 4 dpf siblings and *rb1*$^{smu8/smu8}$ mutants (left). The white ovals indicate the thymus region. The white arrows indicate proliferative T cells. Scale bars: 20 μm. The graph shows the percentages of *rag2:DsRed*$^{+}$ T cells that incorporate BrdU (mean± s.e.m.; ns, not significant; *n*=10). (B) Double staining of TUNEL/*rag2-dsRed* shows TUNEL incorporation by T-cells in 4 dpf siblings and *rb1*$^{smu8/smu8}$ mutants. The broken line outlines the thymus region. The white arrows indicate apoptotic T cells. Scale bar: 50 μm. The graph shows the percentages of *rag2:DsRed*$^{+}$ T cells that incorporate TUNEL (mean±s.e.m.; ***P<0.001; *n*=9). (C) Confocal images of T cells (red) exhibiting cell apoptosis (overlap of TUNEL staining) of siblings and *rb1*$^{smu8/smu8}$ mutant embryos injected with control or pTol-*lck:rb1* plasmid at 5 dpf. Scale bars: 50 μm. The graph shows the percentages of T cells (red) exhibiting cell apoptosis (overlap of TUNEL staining) in siblings and *rb1*$^{smu8/smu8}$ mutant embryos injected with control or pTol-*lck:rb1* plasmid (mean±s.e.m.; ***P<0.001; *n*=10).

whether P53 is required to limit apoptosis after Rb1 loss, we examined the expression of *rag1* in *tp53*$^{M214K/M214K}$ and *tp53*$^{M214K/M214K}$ *rb1*$^{smu8/smu8}$ zebrafish double mutants. We found that the expression of *rag1* was not restored in embryos with *rb1* and *p53* double-knockout larvae at 5 dpf (Fig. 4A). Consistently, the expression of *p53* and its downstream targets [*p21* (*cdkn1a*) *mdm2* and *ccng1*] in the thymus region of *rb1* mutants remained unchanged (Fig. 4A), indicating that early T-lymphocyte deficiency in *rb1* knockout zebrafish mutants is independent of the *p53* pathway.

Caspases, a family of cysteine proteases, are highly conserved throughout vertebrates and are mostly known as executioners of apoptosis (Chowdhury et al., 2008). To determine whether the Rb1 loss-induced apoptosis depends on the caspase cascade, we used the pan-caspase inhibitor Z-VAD-FMK (Vandenabeele et al., 2006) to rescue the lymphocyte apoptosis in the mutants. We found that Z-VAD-FMK can significantly restore early T lymphocyte numbers in *rb1*$^{smu8/smu8}$ mutants (Fig. 4B), suggesting that Rb1 knockout-induced apoptosis is mediated by caspases. Furthermore, we found that both the gene expression and the protein product activity of *casp3* were markedly increased in thymus region in mutants compared with siblings (Fig. 4C). These results reveal that the apoptosis of early T lymphocytes in Rb1-deficient mutants is dependent on the activation of caspase 3.

Apoptosis of immature T lymphocytes of *rb1*$^{smu8/smu8}$ mutants is *e2f1* mediated

Previous studies have suggested that E2F1 is able to trigger cell apoptosis (Lin et al., 2001), and *e2f1*$^{-/-}$ mice exhibit an excess of mature T lymphocytes owing to apoptosis deficiency (Field et al., 1996). We first detected the expression level of *e2f1* mRNA in thymus region and found it specifically upregulated in *rb1*$^{smu8/smu8}$ larvae but not in wild-type larvae (Fig. 5A). To investigate whether *e2f1* is essential for cell apoptosis regulation during T lymphocyte maturation in zebrafish, knockdown experiments were conducted by injection of *e2f1* splice antisense morpholino (MOsp) into wild-type embryos (Fig. 5B,C). The MOsp is predicted to bind to the exon-intron boundary of *e2f1* mRNA to block its expression, and the expression level of *e2f1* is indeed downregulated in morphants (Fig. 5B). As expected, the number of *rag2:DsRed*$^{+}$ T cells was increased in the thymus of *e2f1*-knockdown morphants (Fig. 5C). These data suggest that *e2f1* negatively regulates T cell numbers in zebrafish. To further test whether E2F1 acts downstream of Rb1 in T lymphocyte development, we performed *e2f1* knockdown experiments in *rb1*$^{smu8/smu8}$ embryos to examine the T-cell development. After *e2f1* knockdown, *rag2:DsRed*$^{+}$ T cells were partially restored owing to the reduced apoptotic T-cell numbers in *rb1*$^{smu8/smu8}$ mutants (Fig. 5D-F). Consistently, T-cell marker genes [*rag2*, *tcra* (*trac*), *tcrb2* and *cd3* (*ighv1-2*)] were upregulated in *e2f1*

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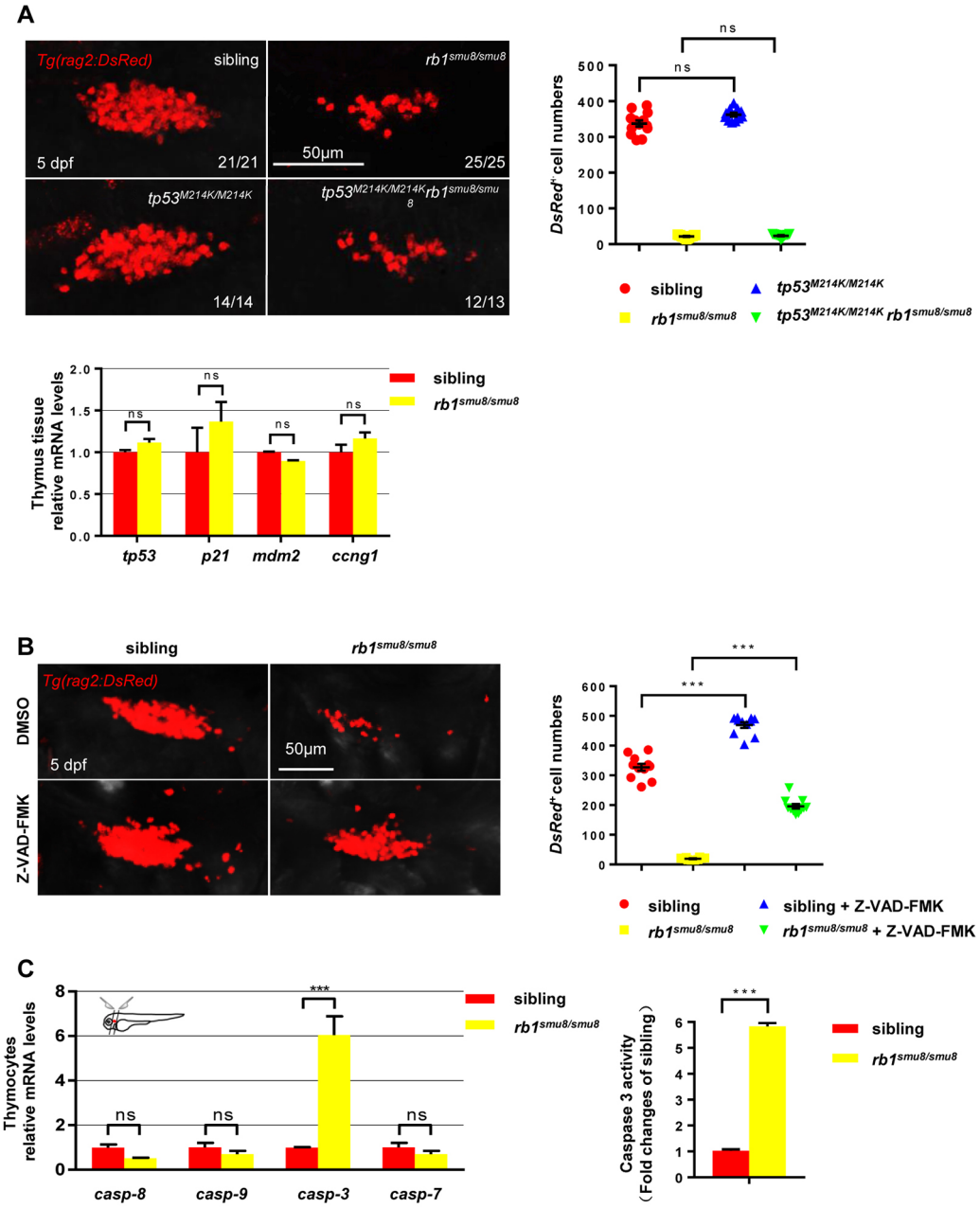

Fig. 4. *rb1* deficiency induced apoptosis in a caspase-dependent manner. (A) Confocal images of *rag2:DsRed* cells in the thymus of the siblings, *rb1*$^{smu8/smu8}$ mutants, *tp53*$^{M214K/M214K}$ and *tp53*$^{M214K/M214K}$*rb1*$^{smu8/smu8}$ double mutants at 5 dpf. Scale bars: 50 µm. Quantification of *DsRed*$^{+}$ cell numbers in the thymus (right). Expression of *tp53*, *p21*, *mdm2* and *ccng1* in thymocytes excised from siblings and *rb1*-deficient embryos at 5 dpf (bottom) (mean±s.e.m.; ns, not significant; *n*=11). (B) Confocal images of *rag2:DsRed* cells in the thymus of DMSO-treated siblings, DMSO-treated *rb1*$^{smu8/smu8}$ mutants, Z-VAD-FMK-treated siblings and Z-VAD-FMK-treated *rb1*$^{smu8/smu8}$ mutants at 5 dpf. Scale bars: 50 µm. Quantification of *DsRed*$^{+}$ cell numbers in the thymus of sibling embryos and *rb1*$^{smu8/smu8}$ mutants (right) (mean±s.e.m.; ***P<0.001; n=10). (C) Expression of caspase mRNA in thymocytes excised from siblings and *rb1*-deficient embryos at 5 dpf (mean±s.e.m.; *P<0.05; ***P<0.001; ns, not significant; n=30). The activity of caspase 3 in siblings and *rb1*$^{smu8/smu8}$ embryos is expressed as the fold change compared with siblings (mean±s.e.m.; ns, not significant; n=10).

morphants and, as expected, the expression of these markers was downregulated in *rb1*$^{smu8/smu8}$ mutants and could be restored by *e2f1* knockdown in *rb1*$^{smu8/smu8}$ mutants (Fig. 5G). These data suggest that *e2f1* knockdown could partially restore T-cell differentiation. In addition, we also examined the expression of *casp3* in *e2f1* morphants and showed that it is downregulated (Fig. 5G). Moreover, the elevated *casp3* expression in *rb1*$^{smu8/smu8}$ mutants could be restored to normal by *e2f1* knockdown (Fig. 5G). The above results reveal that the apoptosis of T lymphocytes in *rb1*$^{smu8/smu8}$ mutants is E2F1 mediated, and *casp3* may act downstream of Rb1-E2F1 axis-mediated T-lymphocyte development.

Rb1-E2F1-mediated immature T lymphocytes apoptosis via caspase 3

Previous studies have shown that E2F1 can upregulate the transcription of *casp3* to induce cell apoptosis (Müller et al., 2001). To further determine whether caspase 3 acts as an executor in Rb1-E2F1 axis-mediated T-lymphocyte development, we overexpressed

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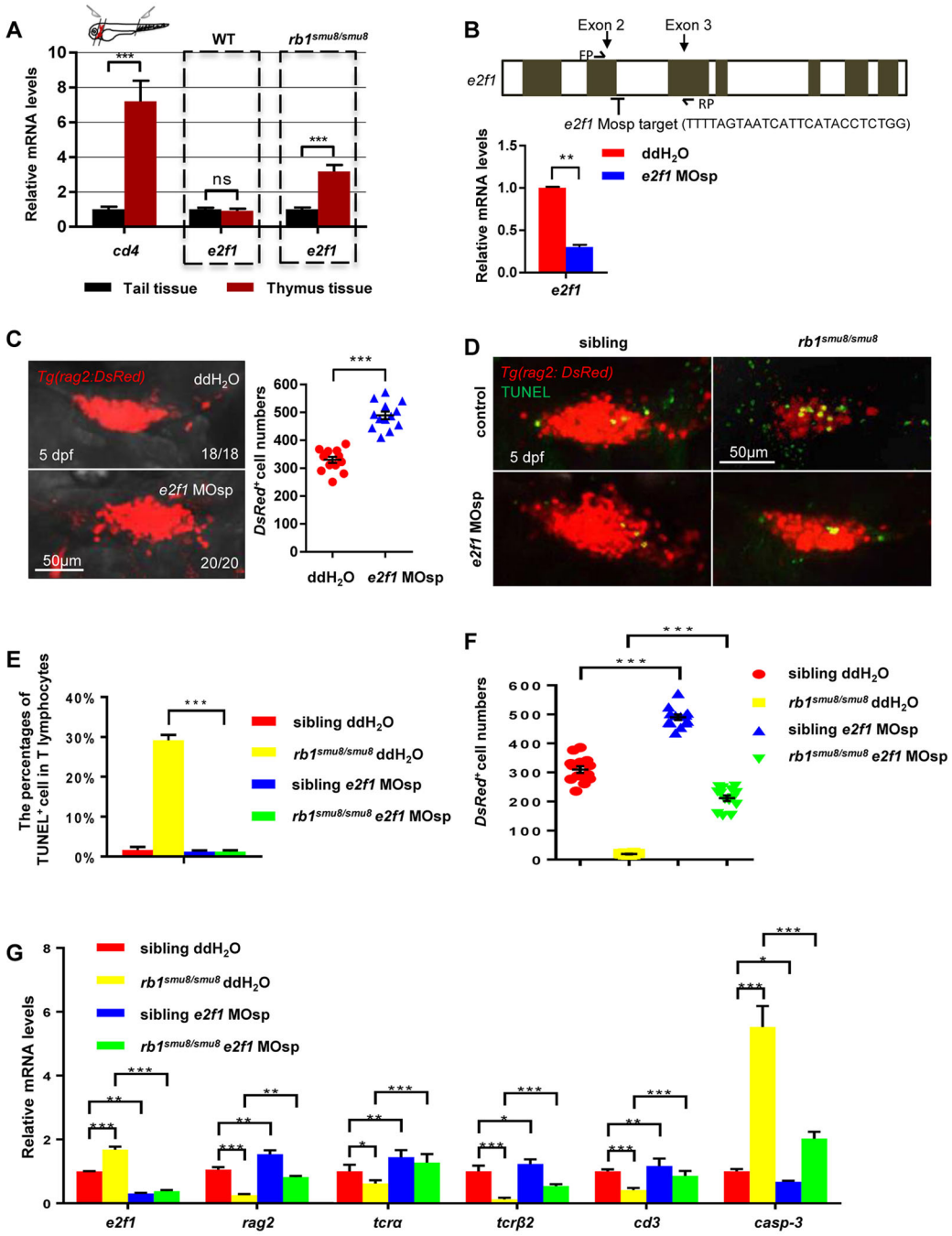

Fig. 5. The excessive apoptosis of early T lymphocytes in the thymus of *rb1*$^{smu8/smu8}$ mutants is *e2f1* mediated. (A) Relative expression of *e2f1* in excised thymus and tail tissue from 5 dpf embryos by qPCR analysis (mean±s.e.m.; ***P<0.001; ns, not significant; n=30). (B) The zebrafish *e2f1* gene structure. Exons are indicated by grey boxes. Location and sequence of the splice morpholino (MOsp) target site for the *e2f1* gene. FP/RP, *e2f1* qPCR forward/reverse primers. Relative expression of *e2f1* in controls and *e2f1* morphants at 5 dpf by qPCR analysis (mean±s.e.m.; **P<0.01; n=30). (C) Confocal images of *rag2:DsRed* cells in the thymus of wild-type embryos injected with double distilled H_2O or *e2f1* MOsp at the one-cell stage with *rag2:DsRed*$^+$ cell number measured at 5 dpf. Scale bars: 50 µm. Quantification of *rag2-DsRed*$^+$ cells (right panel) (mean±s.e.m.; ***P<0.001; n=13). (D) Confocal images of T cells (red) exhibiting cell apoptosis (overlap of TUNEL staining) in siblings and *rb1*$^{smu8/smu8}$ mutant embryos injected with double distilled H_2O or *e2f1* MOsp at 5 dpf. Scale bars: 50 µm. (E) Percentages of T cells (red) exhibiting cell apoptosis (overlap of TUNEL staining) in siblings and *rb1*$^{smu8/smu8}$ mutant embryos injected with double distilled H_2O or *e2f1* MOsp at 5 dpf (mean±s.e.m.; ***P<0.001; n=20). (F) Quantification of *rag2-DsRed*$^+$ cell shown in D (mean±s.e.m.; ***P<0.001; n=10). (G) qPCR analysis of *e2f1*, *rag2*, *tcra*, *tcrb*, *cd3* and *casp3* expression in mutants injected with double distilled H_2O or *e2f1* MOsp at 5 dpf (mean±s.e.m.; ***P<0.001, **P<0.01 *P<0.05; n=30).

e2f1 in *rb1*$^{smu8/smu8}$ T lymphocytes and examined *casp3* expression. We found that the T lymphocyte marker *rag1* was downregulated by *e2f1* overexpression, which mimics the low *rag1* levels in *rb1*-deficient embryos (Fig. 6A). The expression of *casp3* was upregulated by *e2f1* overexpression and was further elevated in *rb1*-deficient larvae (Fig. 6A). Meanwhile, Z-VAD-FMK was used to inhibit caspase activity in *e2f1*-overexpressed/*rb1*-deficient embryos, and T lymphocyte numbers were counted. As expected,

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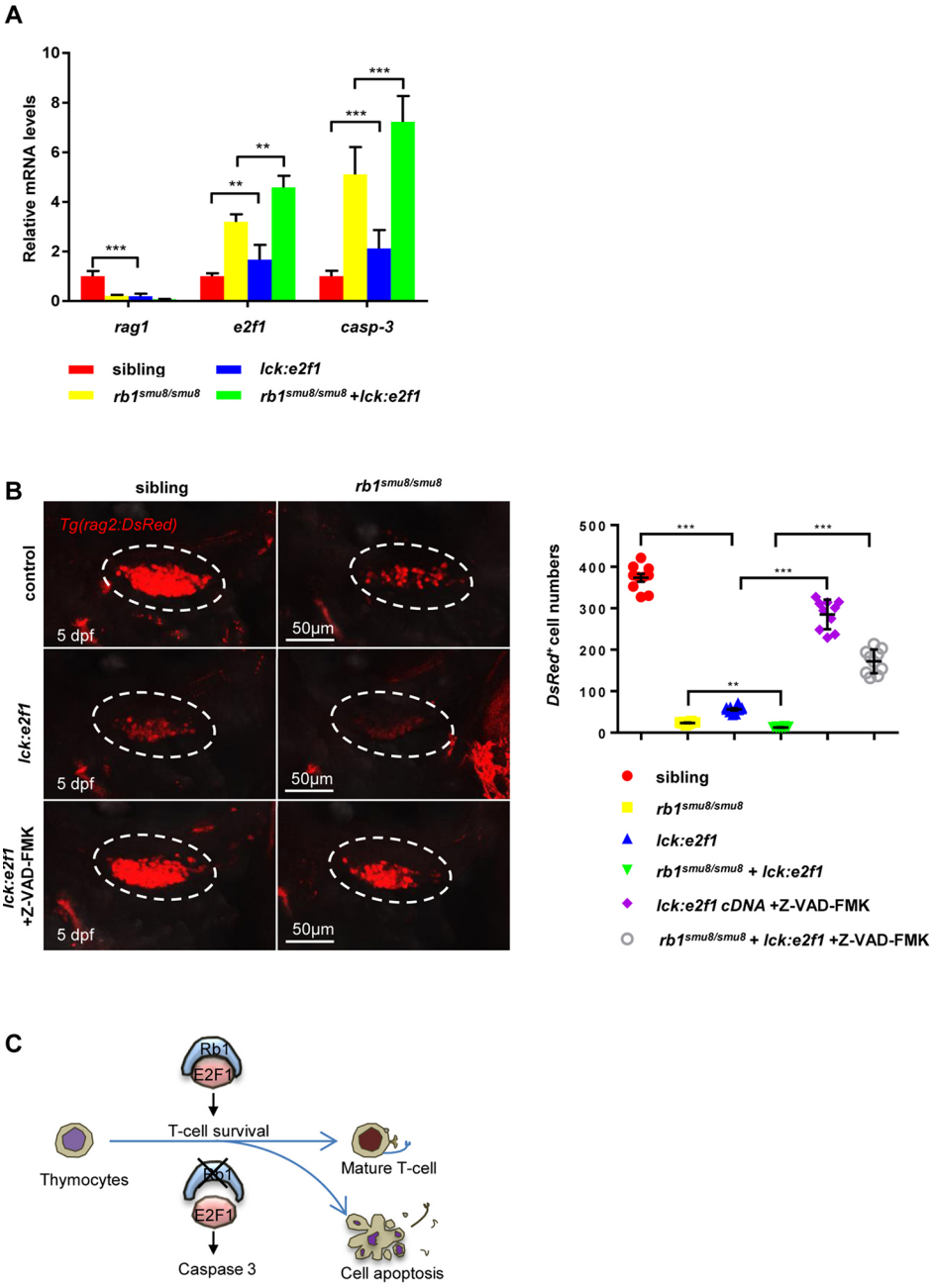

Fig. 6. E2F1 induced apoptosis of immature T lymphocytes by promoting the expression of *casp3*. (A) Relative expression of *rag1*, *e2f1* and *casp3* in siblings and mutants injected with control or pTol-*lck:e2f1* plasmid (mean±s.e.m.; ***P<0.001; **P<0.01; n=30). (B) Confocal images of T cells (red) in siblings and in *rb1*$^{smu8/smu8}$ mutants injected with double distilled H_2O or *lck-e2f1* plasmid, followed by Z-VAD-FMK or DMSO treatment. The white circles indicate the thymus region. Scale bars: 50 µm. Quantification of *rag2-DsRed* cell (mean ±s.e.m.; ***P<0.001, **P<0.01; n=10) (C) A schematic diagram of Rb1-E2F1-caspase 3 axis-regulated apoptosis during T lymphocyte maturation.

overexpressing *e2f1* can downregulate the number of *rag2:DsRed*$^{+}$ T lymphocytes, and the T lymphocyte loss can be recovered by suppressing the caspases activity (Fig. 6B), suggesting that caspase 3 acts downstream of E2F1 during the apoptosis of immature T cells in *rb1*$^{smu8/smu8}$ mutants. In summary, we demonstrated that Rb1 inhibits apoptosis during early immature T-lymphocyte development by repressing the activity of E2F1 to downregulate *casp3* expression (Fig. 6C).

DISCUSSION

To investigate the function of Rb1 in the regulation of T-cell development, we generated Rb1-deficient zebrafish. Zebrafish *rb1*$^{smu8/smu8}$ mutants display a significantly decreased number of T cells, with other hematopoietic cell development being largely unaffected. The decreased T cell number appears to be due to excessive apoptosis that occurs in the immature T cells, which is mediated by caspase 3 but independent of P53. We further showed that E2F1 knockdown could rescue Rb1 deficiency-induced apoptosis, suggesting a crucial role for the Rb1-E2F1-caspase axis in the regulation of immature T-lymphocyte apoptosis.

Our data suggest that during the embryonic stages of T-cell development in zebrafish, Rb1 inhibits apoptosis by repressing the activity of E2F1 and downstream caspase activation. It is possible that, under normal circumstances, in the apoptosis of nonfunctional or self-reactive thymocytes, the dissociated E2F1 from Rb1 plays a crucial role in promoting *casp3* expression. However, when E2F1 is overexpressed or there are insufficient Rb1 to bind up all the E2F1, derepression of E2F1 would promote inappropriate apoptosis in early immature thymocytes. It is worth unveiling the mechanism, but there are several challenges in the current study. Antibodies specific for zebrafish T-cell sub-populations have yet to be developed, which makes the identification of distinct developmental stage T cells

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impossible at present. Nonetheless, we believe that this problem is likely to be solved with the creation of new transgenic zebrafish.

Rb1-null mice studies showed that Rb1 loss induced not only disturbed proliferation, but also excess apoptosis in the neural system and deregulated the maturation of erythrocytes (Chau and Wang, 2003; Lee et al., 1992). Rb1 absence also has been reported to be harmful to many cellular processes, including differentiation (Korenjak and Brehm, 2005; McClellan and Slack, 2007), survival (Delston and Harbour, 2006; Hallstrom and Nevins, 2009), senescence (Ben-Porath and Weinberg, 2005; Liu et al., 2004) and genome stability (Knudsen et al., 2006). However, none of these studies has underlined the exact function of Rb1 in the development of T lymphocytes. This may be because zebrafish develop rapidly *ex utero* (Langenau and Zon, 2005) and lymphopoiesis occurs in the thymus by 3 dpf (Jagannathan-Bogdan and Zon, 2013). Compared with Rb1-null mice, which die before E16 with multiple defects (Lee et al., 1992), Rb1-null zebrafish can survive to 15 dpf. Interestingly, the thymic cellularity of *E2f1*$^{-/-}$ mice was noticeably increased at 4-6 weeks. These data indicate that Rb1 may also be necessary for T-cell development in mice through repression of *E2f1*.

To address the issue of Rb1 specificity in regulating T-cell development more adequately, we examined the expression of *rb1* in different hematopoietic cell types and found that *rb1* is more abundant in T lymphocytes than in other blood cells (Fig. S2H). Likewise, *e2f1* levels are much higher in the thymus compared with the tail region when Rb1 is mutated (Fig. 5A). These data indicate that Rb1-E2F1 pathway plays a crucial role in T-cell development in the thymus, in which 95% of immature thymocytes are eliminated via apoptosis (Kappler et al., 1987). We believe that a relative high level of Rb1 in developing T cells is crucial for inhibiting *e2f1* activity, thereby preventing normal developing T cells from inappropriate apoptosis.

As shown previously, both Rb1-deficient mice and Rb1-deficient zebrafish display severe neuronal defects (Clarke et al., 1992; Gyda et al., 2012; Lee et al., 1992). Consistent with previous findings, we also found that *rb1* is highly expressed in the brain tissue (data not shown) and neuron apoptosis is significantly increased in *rb1*$^{smu8/smu8}$ (increased neuronal apoptosis could be rescued by a caspase inhibitor and *casp3* was upregulated in brain in *rb1*$^{smu8/smu8}$, data not shown), suggesting that Rb1 plays a similar role in neuron and T-cell development. Interestingly, during both T-cell development and neurogenesis, a large numbers of undesired cells must undergo apoptosis. We speculate that Rb1 may play an essential role in preventing desired cells from inappropriate apoptosis.

Chromosome instability (CIN) and aneuploidy are a common feature of tumour cells, and studies have shown that Rb1 inactivation could promote CIN and aneuploidy (Manning et al., 2010). Clinically, *RB1* deletions are frequently associated with additional acquired chromosomal copy number changes in individuals with CLL (Ouillette et al., 2011). This mis-segregation of chromosomes causes eventual death in cells that lack Rb1 function. However, we suspect that when thymocytes are not regulated, such genomic changes potentially promote the evolution of CLL. Combined with our finding that immature lymphocytes that lack Rb1 have enhanced cell apoptosis, removal of Rb1-deficient cells may be important for organ homoeostasis when treating CLL.

In summary, our results provide the first functional assay of Rb1 in early T-cell development and show that Rb1 inhibits E2F1 to trigger the caspase cascade during early T-lymphocyte maturation. Given the fact that somatic deletion of *RB1* is the most frequent chromosomal abnormality in CLL, elucidating the mechanism behind the regulation of immature T-cell apoptosis regulation by Rb1 provides an intriguing link between tumour suppression and lymphatic system development that needs to be further investigated.

MATERIALS AND METHODS

Fish maintenance

Zebrafish were maintained at 28.5°C in a 14 h light and 10 h dark cycle. Embryos were collected by natural spawning and raised at 28.5°C. To prevent the formation of melanin pigment, embryos were incubated in egg water containing 0.045% 1-phenyl-2-thiourea (PTU, Sigma, P7629) after gastrulation stage. The embryos were collected at the desired stages (Westerfield, 2000). The following strains were used: AB, *tp53*^{M214K} (Berghmans et al., 2005), *Tg(rag2:Dsred)* (Ma et al., 2012), *Tg(mpeg1:loxP-DsRedx-loxP-GFP)* (Ellett et al., 2011), *Tg(lyz:Dsred)* (Hall et al., 2007), *Tg(globin:Dsred)* and *rb1*$^{smu8/smu8}$ mutants.

Whole-mount *in situ* hybridization

Synthesis of digoxigenin-labelled antisense RNA probes and whole mount *in situ* hybridization were performed as described previously (Jin et al., 2016; Liu et al., 2017). The probes were listed as follows: *rag1*, *myb*, *lck*, *ikaros*, *foxn1*, *pu.1*, *lyz*, *mfap4*, *gata1* and *βe1*.

Western blotting

Western blotting was performed as described previously (Huang et al., 2002). The anti-Rb1 antibody was obtained from Proteintech (17218-1-AP).

Overexpression of *rb1* or *e2f1* in T cells

The coding sequences of zebrafish *rb1* and *e2f1* were amplified by PCR and spliced into the *lck* or *rag2* promoter-containing pTol vector using *Xma*I/*Bam*HI or *Age*I/*Bam*H1 digestion, respectively. The *lck* and *rag2* promoters have been described previously (Jessen et al., 2001; Langenau et al., 2004). pTol-*lck:rb1*, pTol-*lck:e2f1* or *pTol-rag2:rb1* with transposase mRNA were injected into one-cell stage AB embryos at a dose of 100 pg/embryo.

BrdU labelling and double staining

Embryos at 4 dpf stage were incubated in 10 mmol/l bromodeoxyuridine (BrdU, Sigma-Aldrich, B9285) solution (0.5% DMSO in egg water) for 4 h and subsequently fixed in 4% paraformaldehyde. After 30-min treatment with 2 N HCl, the embryos were stained using primary mouse anti-BrdU (Roche, 10875400; 1:50, at 4°C overnight) and rabbit anti-DsRed (Clontech, 632496; 1:400, at 4°C overnight) antibodies, and finally were visualized with Alexa Fluor 555 donkey anti-mouse (Invitrogen, A31572) and Alexa Fluor 488 donkey anti-rabbit (Invitrogen, A21206) antibodies.

TUNEL labelling and double staining

The paraformaldehyde-fixed embryos (5 dpf stage) were further incubated in a PBST solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 15 min followed by three rinses in PBST. The embryos were subsequently soaked in the terminal deoxynucleotidyl transferase dUTP nick end labelling mix using the *in situ* cell death detection kit (Roche, 12156792001) at 37°C overnight and stained using the anti-DsRed antibody.

Quantitative real-time PCR

Total RNA extraction and complementary DNA synthesis were performed as described previously (Lin et al., 2016). Quantitative reverse-transcription PCR (qRT-PCR) was performed using the light cycler Nano Real-time PCR system (Roche) with an SYBR Green Master mix (Roche, 06402712001). The housekeeping gene *ef1a* was used as the internal control. A least 30 embryos were included in each experiment. The primer sequences are described in Table S1.

Mutant identification

rb1$^{smu/smu8}$ mutants were genotyped by PCR followed by *Bcl*I (Thermo, ER0722) digestion. The wild-type PCR products were digested using *Bcl*I into two fragments of 198 bp and 176 bp, respectively, whereas mutant PCR products were resistant to the *Bcl*I digestion. Primers for *rb1* genotyping

were as follow: FP, 5′-GCCACTGCTAAACACTAAAGA-3′; RP, 5′-GCTCCATGCCAGCAATAAAA-3′.

Mopholino oligonucleotide injection

The design and injection of *e2f1* MOs was performed as previously reported (Bill et al., 2009). *e2f1* MOsp (5′-TTTTAGTAATCATTCATACCTCTGG-3′) targeting protein translation were obtained from Gene Tools and injected into zebrafish embryos at the one-cell stage (0.5 pmol per embryo). The number of T lymphocytes was quantified at 5 dpf.

Drug treatments

Z-VAD-FMK (V116, 200 µM) was purchased from Sigma-Aldrich and dissolved in egg water with DMSO.

Caspase 3 activity assay

Caspase 3 activity was determined using the Caspase 3 Activity Kit (Beyotime, C1115). Ten thymus excised from the larvae were collected and pooled as one sample. The larvae were washed twice with phosphate-buffered saline (PBS) and then homogenized in 100 µl of lysis buffer on ice for 5 min. The lysate was centrifuged at 16,000 ***g*** at 4°C for 15 min. The supernatants were collected and immediately measured for total protein concentration and caspase 3 activity. For the caspase 3 activity assay, 10 µl of supernatant was placed in a 96-well plate containing 80 µl reaction buffer and 10 µl of caspase 3 substrate (Ac-DEVD-pNA). The plate was incubated at 37°C in the dark for 30 min, and enzyme activity was determined through measuring the optical density of each sample at 405 nm using TECAN infinite M200 Absorbance Reader. Total protein concentration was determined using a Bradford assay (Beyotime, P0006).

Statistical methods

The calculated data were recorded and analysed using prism software. The unpaired two-tailed Student's *t*-test for comparisons between two groups and one-way analysis of variance (ANOVA; with Bonferonni or Dunnett T3 post-test adjustment) among multiple groups. $P<0.05$ was deemed significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.Z., W.Z., Y.Z.; Methodology: Z.Z., W.L., L.Z., Z.H., X.C., N.M., Y.Z.; Software: Z.Z., W.L., L.Z., X.C.; Validation: Z.Z., W.L., L.Z., Y.Z.; Formal analysis: Z.Z., W.L., L.Z., Z.H., N.M., J.X., W.Z., Y.Z.; Investigation: Z.Z., W.L., L.Z.; Resources: Z.Z., X.C., Y.Z.; Data curation: Z.Z., L.Z., Z.H., N.M., J.X., W.Z., Y.Z.; Writing - original draft: Z.Z., Y.Z.; Writing - review & editing: W.L., Z.H., J.X., W.Z., Y.Z.; Visualization: Z.Z., X.C., W.Z., Y.Z.; Supervision: W.Z., Y.Z.; Project administration: W.Z., Y.Z.; Funding acquisition: W.L., W.Z., Y.Z.

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Supplementary information

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