TOPOISOMERASE1α Acts through Two Distinct Mechanisms to Regulate Stele and Columella Stem Cell Maintenance[1][OPEN]

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TOPOISOMERASE1 (TOP1), which releases DNA torsional stress generated during replication through its DNA relaxation activity, plays vital roles in animal and plant development. In Arabidopsis (*Arabidopsis thaliana*), TOP1 is encoded by two paralogous genes (*TOP1α* and *TOP1β*), of which *TOP1α* displays specific developmental functions that are critical for the maintenance of shoot and floral stem cells. Here, we show that maintenance of two different populations of root stem cells is also dependent on *TOP1α*-specific developmental functions, which are exerted through two distinct novel mechanisms. In the proximal root meristem, the DNA relaxation activity of TOP1α is critical to ensure genome integrity and survival of stele stem cells (SSCs). Loss of *TOP1α* function triggers DNA double-strand breaks in S-phase SSCs and results in their death, which can be partially reversed by the replenishment of SSCs mediated by *ETHYLENE RESPONSE FACTOR115*. In the quiescent center and root cap meristem, *TOP1α* is epistatic to *RETINOBLASTOMA-RELATED* (*RBR*) in the maintenance of undifferentiated state and the number of columella stem cells (CSCs). Loss of *TOP1α* function in either wild-type or *RBR RNAi* plants leads to differentiation of CSCs, whereas overexpression of *TOP1α* mimics and further enhances the effect of *RBR* reduction that increases the number of CSCs. Taken together, these findings provide important mechanistic insights into understanding stem cell maintenance in plants.

DNA TOPOISOMERASE1 (TOP1) is a key eukaryotic nuclear enzyme that regulates the topology of DNA during replication, transcription, and chromatin remodeling (Liu and Wang, 1987). TOP1 relaxes torsional tension by nicking DNA followed by controlled rotation of the broken DNA strand around the intact strand and resealing of the nick (Champoux, 2001; Wang, 2002). Inhibition of TOP1-mediated DNA resealing step with camptothecin (CPT), an anticancer alkaloid isolated from plants carrying CPT-resistant point mutations in TOP1 (Sirikantaramas et al., 2008), induces DNA double-strand breaks (DSBs) and in some cases cell death (Hsiang et al., 1985; Hsiang and Liu, 1988; Porter and Champoux, 1989). High CPT doses might also lead to incomplete DNA replication and persistent fork stalling, causing DSBs and cell death (Koster et al., 2007; Ray Chaudhuri et al., 2012). Interestingly, although there is considerable diversity in the amino acid sequences of TOP1 proteins of plants and animals, the same compensatory mutation found in CPT-producing plants also contributes to CPT resistance in CPT-resistant human cancer cells (Fujimori et al., 1995; Sirikantaramas et al., 2008), suggesting that the CPT-interacting residues in TOP1 are conserved across kingdoms.

The developmental functions of TOP1 in animals have been difficult to study because *TOP1* knockouts are embryonic lethal (Lee et al., 1993; Morham et al., 1996). Nevertheless, using RNA interference (RNAi) and cosuppression techniques, a recent study showed that *TOP1* in *Caenorhabditis elegans* may function in morphogenesis, stem cell niche specification, normal life span, and growth control (Lee et al., 2014). In plants,

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the presence of TOP1 is also essential. The disruption of the two TOP1 encoding genes, *TOP1α* and *TOP1β*, in Arabidopsis (*Arabidopsis thaliana*) resulted in seedling lethality (Takahashi et al., 2002), indicating that TOP1α and TOP1β are redundantly required for the survival of Arabidopsis. Interestingly, only *top1α* mutants displayed obvious defects associated with organization of shoot, floral, and root meristems (Laufs et al., 1998; Takahashi et al., 2002; Graf et al., 2010; Liu et al., 2014), suggesting that TOP1α has specific developmental functions. Accordingly, TOP1α was found to regulate stem cell maintenance in shoots and flowers despite a lack of evidence from cell type-specific measurements (Liu et al., 2014). However, roles in the root remain to be elucidated.

Here, we show that TOP1α contributes significantly to the maintenance of stem cells in the Arabidopsis root. More specifically, we demonstrate that TOP1α acts through two distinct novel mechanisms to regulate the maintenance of stele stem cells (SSCs) and columella stem cells (CSCs). TOP1α is required for the survival of SSCs and regulates the differentiation state and number of CSCs.

RESULTS

Both *TOP1α* and *TOP1β* Are Transcribed in the Root Meristem, But Only TOP1α Displays Specific Functions in Root Development

To gain insight into the potential roles of *TOP1α* and *TOP1β* in the Arabidopsis root, we generated promoter-GUS transcriptional fusions using the intergenic sequence upstream of *TOP1α* or *TOP1β* and examined their expression patterns in roots of representative transgenic lines. Both *TOP1α::GUS* and *TOP1β::GUS* were found to have preferential expression in the root meristem, including the quiescent center (QC) and the surrounding root stem cells (Fig. 1, A and B), suggesting that *TOP1α* and *TOP1β* have overlapping expression and possibly redundant functions in the root stem cell niche.

Since *TOP1α* and *TOP1β* are tandemly arrayed in the Arabidopsis genome (Fig. 1C), it is impossible to combine loss-of-function mutations in both genes via genetic crosses to investigate and determine functional redundancy between them. Therefore, we used the root meristem-specific *ROOT CLAVATA HOMOLOG1* (*RCH1*)

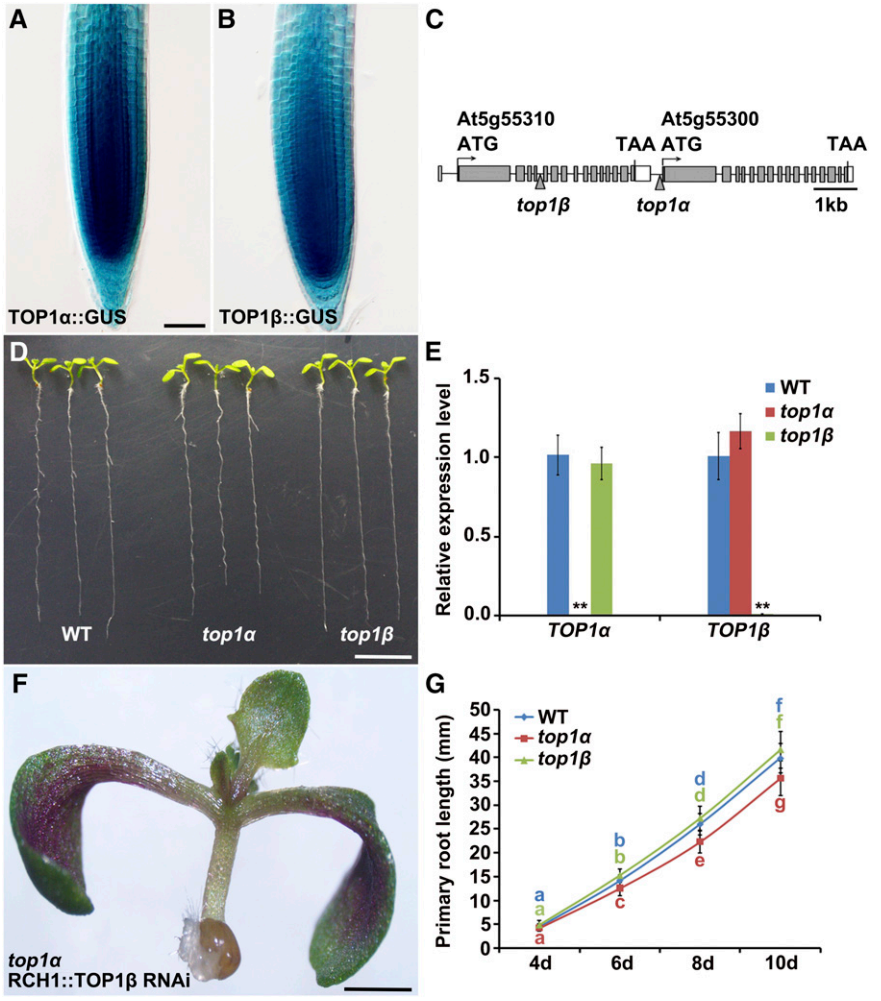

Figure 1. Both *TOP1α* and *TOP1β* are transcribed in the root meristem, but only TOP1α displays specific functions in root development. A and B, Expression (stained in blue) of *TOP1α::GUS* (A) and *TOP1β*::GUS (B) in wild-type root tips. Bar = 50 μm. C, A schematic diagram showing tandemly arrayed *TOP1α* and *TOP1β* genomic regions. Gray and white boxes represent exons and 3′-untranslated regions, respectively, and horizontal lines indicate introns and 5′-untranslated regions. Arrowheads point to the T-DNA insertion sites in *top1α* and *top1β*. ATG, Transcription start site; TAA, stop codon. D, Phenotypes of 10-d-old wild-type, *top1α*, and *top1β* seedlings. Bar = 1 cm. E, qRT-PCR analysis of *TOP1α* and *TOP1β* transcription in roots of wild-type, *top1α*, and *top1β* seedlings. Transcript levels of *TOP1α* and *TOP1β* in wild-type roots were set to 1. Error bars represent SD from three independent experiments. $^{**}P < 0.01$, *t* test. F, A *top1α* seedling carrying the *RCH1::TOP1β RNAi* transgene. Bar = 1 cm. G, Time-course analysis of root lengths of wild-type, *top1α*, and *top1β* seedlings. Measurements were performed on the indicated days. Error bars represent SD ($n > 20$). Bars with different letters are significantly different at $P < 0.01$, *t* test.

(Casamitjana-Martínez et al., 2003) promoter to direct RNAi-mediated down-regulation of *TOP1β* (Supplemental Fig. S1, A and B) in a *top1α* null mutant (Fig. 1, C and E), which was isolated together with a *top1β* null mutant (Fig. 1, C and E) from the SALK T-DNA collection (Alonso et al., 2003). We found no statistical difference between root growth of wild-type plants and *top1β* mutants (Fig. 1, D and G), but root growth in *top1α* was significantly reduced when compared to the wild-type control and *top1β* (Fig. 1, D and G). This phenotype was dramatically enhanced by *RCH1::TOP1β RNAi*-mediated down-regulation of *TOP1β* (Fig. 1F; Supplemental Fig. S1, D and E), resulting in a rootless phenotype and as expected without leading to the loss of the aerial parts (Fig. 1F; Supplemental Fig. S1, D and E). Taken together, these findings suggest that (1) there is a certain level of functional overlap between *TOP1α* and *TOP1β*, which helps maintain the root; (2) in the presence of *TOP1α*, *TOP1β* is dispensable in the root; and (3) *TOP1α*, but not *TOP1β*, has specific developmental functions in the root.

TOP1α Is Required Cell-Autonomously for the Survival of SSCs

Mutations in *TOP1α* were reported to trigger cell death in the root meristem and affect its organization (Graf et al., 2010), but a detailed phenotypic analysis at the tissue and cell levels is still missing. To close this gap, we performed confocal microscopic analysis of cell death phenotype in wild-type and *top1α* roots using propidium iodide (PI), which is excluded from entering live cells but penetrates into dead cells. We found that loss of *TOP1α* function led to penetration of PI into cells at the position of SSCs (compare Fig. 2, B and C; see Fig. 2A for the position of SSCs), thus confirming the presence of cell death and revealing the identity of dead cells in *top1α* roots. Expression of wild-type *TOP1α* under the control of its native promoter (*TOP1α::TOP1α*) fully complemented the cell death (Fig. 2D) and root growth (Supplemental Fig. S2, A and B) phenotypes of *top1α*, suggesting that *TOP1α* is essential for the survival of SSCs and that the integrity of SSCs is vital for the maintenance of root growth. Moreover, expression of TOP1α in the stele (*SHR::TOP1α*), but not in the cells of the adjacent layer (*SCR::TOP1α*), prevented death of SSCs in *top1α* roots (Fig. 2, E and F), suggesting that TOP1α is required cell-autonomously for survival of SSCs. By contrast, death of SSCs was not observed in *top1β* (Fig. 2G) and *RCH1::TOP1β RNAi* roots (Supplemental Fig. S1C), implying that, in the presence of *TOP1α*, *TOP1β* is dispensable for survival of SSCs.

The DNA Relaxation Activity of TOP1α Is Crucial for the Genome Integrity and Survival of SSCs

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showed that death of SSCs was associated with DNA fragmentation in SSCs of *top1α* roots (compare Fig. 3, A and B), indicating that TOP1α protects the genome integrity of SSCs that is vital for their survival. To determine the underlying molecular mechanism, we next examined whether the DNA relaxation activity of TOP1, encoded by the *TOP1α* gene, is needed to maintain genome integrity of SSCs and ensure their survival. Mild treatment of wild-type roots with 100 nM of the TOP1 inhibitor CPT induced death of SSCs at ~6 h after treatment (Fig. 3C), suggesting that SSCs are particularly sensitive to inhibition of the DNA resealing step catalyzed by TOP1. This phenotype was markedly enhanced at 24 h after CPT treatment (Fig. 3D), at which time death of other types of root stem cells (such as CSCs) was also observed, but to a lower extent (Fig. 3D). Thus, under our conditions, CPT treatment could largely mimic the cell death phenotype observed in *top1α*, indicating that TOP1α-mediated nick religation is needed to maintain genome integrity and, thus, survival of stem cells in the Arabidopsis root. Consistently, TOP1α N871S, which comprises a single amino acid substitution corresponding to the CPT-resistant mutation N722S in human TOP1 (Fujimori et al., 1995), could fully rescue the death phenotype of SSCs in *top1α* mutant roots regardless of the absence or presence of CPT treatment (Fig. 3, E and F). Together, these results suggest that the DNA relaxation activity of TOP1α is needed to relieve DNA torsional stress in SSCs, which otherwise threatens their genome integrity and survival.

Early studies demonstrated that cytotoxicity of CPT is primarily a result of DNA DSBs during the S-phase when the replication fork collides with the cleavage complexes formed by DNA and CPT (Hsiang et al., 1989; Pommier et al., 2003). We therefore investigated whether the death of SSCs in *top1α* was related to replication-mediated DNA DSBs. For this purpose, aphidicolin (APH), a specific inhibitor of replication polymerases that prevents the formation of CPT-induced replication-mediated DNA DSBs was used (Ryan et al., 1991). APH completely prevented the death of SSCs in *top1α* and CPT-treated wild-type roots (Fig. 3, G and H), indicating that genetic and chemical disruption of TOP1α function induces DNA DSBs in S-phase SSCs and consequently their death.

Activation of ERF115-Mediated Replenishment of SSCs Is a Common Response to SSC Death Induced by DNA DSBs

Preferential death of SSCs was also observed after treatment with low levels of DNA DSB-inducing agents such as bleomycin and zeocin (Fulcher and Sablowski, 2009; Heyman et al., 2013; (compare Supplemental Fig. S3, A and B), suggesting that SSCs are particularly vulnerable to DNA DSBs, regardless of their cause. Under the reported conditions, not all SSCs died, and ERF115, a transcription factor of the 122-member ERF family, was found to act redundantly with its homologs to facilitate the replenishment of SSCs, allowing the root

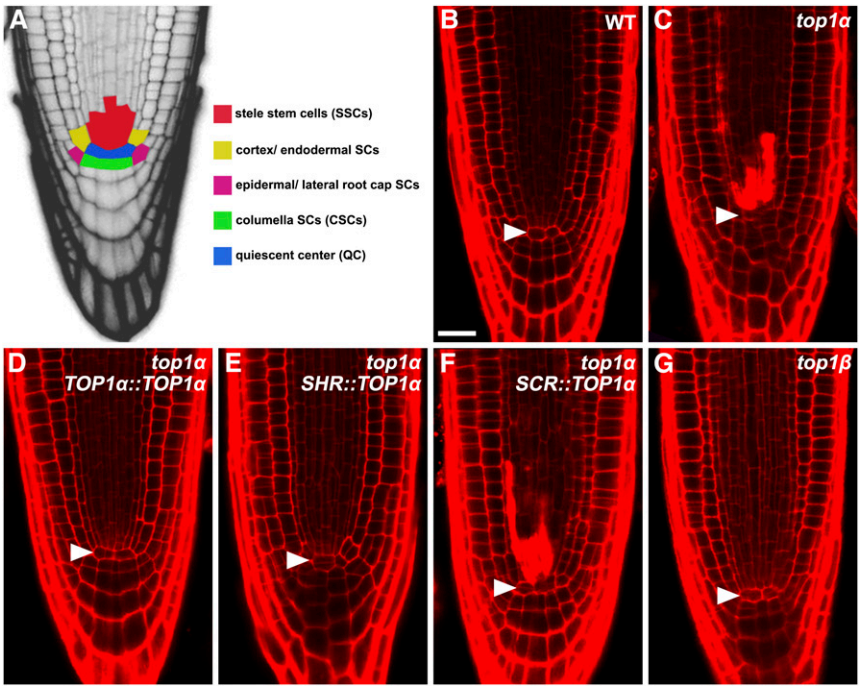

Figure 2. *TOP1α* is required cell-autonomously for the survival of SSCs. A, A schematic medial longitudinal section of the Arabidopsis root tip. The QC and different types of root stem cells are color-coded. B and C, Root tips of wild-type (B) and *top1α* (C) seedlings. D to F, Root tips of *top1α* seedlings carrying the *TOP1α::TOP1α* (D), *SHR::TOP1α* (E), or *SCR::TOP1α* (F) transgene. G, Root tip of a *top1β* seedling. Root cells were counterstained (in red) with PI and imaged with confocal microscopy. PI is excluded from entering live cells but penetrated into dead cells. Arrowheads point to the QC. Bar = 25 μm.

to resume growth after the removal of DNA DSB-inducing agents (Heyman et al., 2013). These findings led us to question whether ERF115 and its homologs also contribute to the replenishment of SSCs and consequently to the maintenance of root growth (as seen in Fig. 1G) in *top1α*, in which SSCs are continuously challenged by DNA DSBs.

Under physiological conditions, the expression of *ERF115*, as indicated by *ERF115* promoter-*GUS* fusions (*ERF115::GUS* and previously reported *pERF115:GUS*; Heyman et al., 2013), was undetectable in the wild-type root (Fig. 4A; Supplemental Fig. S4A). However, it was strongly and specifically activated at the position of SSCs by mild treatment with zeocin (Fig. 4B;

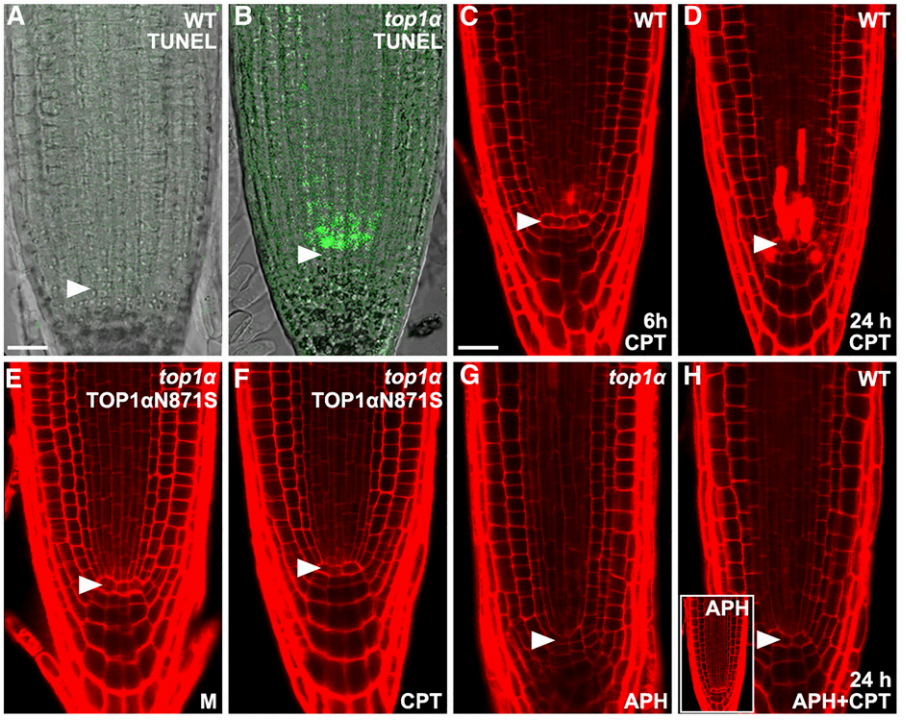

Figure 3. The DNA relaxation activity of TOP1α is crucial for the genome integrity and survival of SSCs. A and B, TUNEL assay of DNA fragmentation (stained in green) in root tip cells of wild-type and *top1α* seedlings. C and D, Root tips of wild-type seedlings treated with 100 nM CPT for 6 h (C) and 24 h (D). E and F, Root tips of *top1α* seedlings carrying the TOP1αN871S transgene, which were mock-treated for 24 h (E) or treated with 100 nM CPT for 24 h (F). G, Root tip of a *top1α* seedling treated with 48 μM APH for 24 h. H, Root tip of wild-type seedlings treated with 48 μM APH (inset) or 48 μM APH and 100 nM CPT for 24 h. Cells were counterstained with PI and imaged with confocal microscopy. Arrowheads point to the QC. M, Mock. Bars = 25 μm.

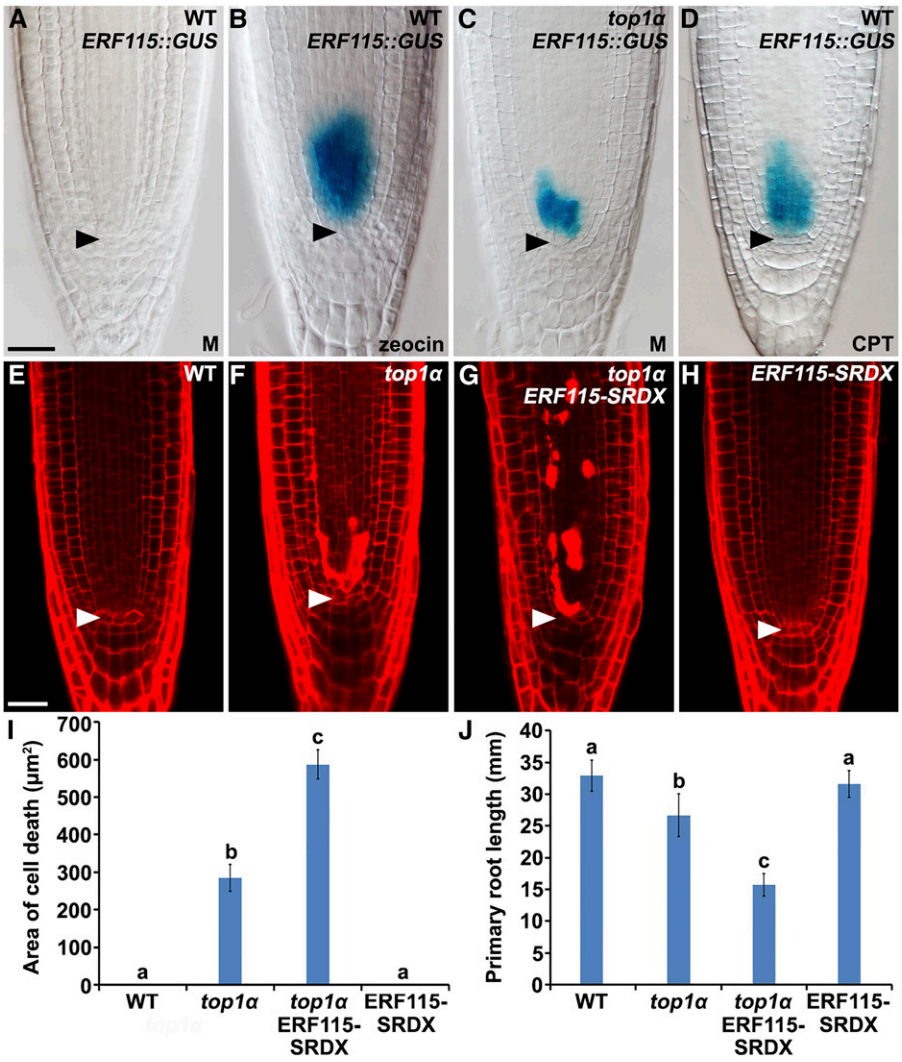

Figure 4. Activation of ERF115-mediated replenishment of SSCs is a common response to SSC death induced by DNA DSBs. A and B, Expression (stained in blue) of *ERF115::GUS* in root tips of wild-type seedlings, which were mock-treated for 24 h (A) or treated with 13 μM zeocin for 24 h (B). C, Expression of *ERF115::GUS* in the root tip of a *top1α* seedling, which was mock-treated for 24 h. D, Expression of *ERF115::GUS* in the root tip of a wild-type seedling, which was treated with 100 nM CPT for 24 h. E to H, Root tips of wild-type (E), *top1α* (F), *top1α ERF115-SRDX* (G), and *ERF115-SRDX* (H) seedlings. I, Quantification of cell death area (μm^2) in roots of 5-d-old wild-type, *top1α*, *top1α ERF115-SRDX*, and *ERF115-SRDX* seedlings. Error bars represent SE ($n > 12$). Bars with different letters are significantly different at $P < 0.01$ (B), *t* test. J, Root lengths of 10-d-old wild-type, *top1α*, *top1α ERF115-SRDX*, and *ERF115-SRDX* seedlings. Error bars represent SD ($n > 20$). Bars with different letters are significantly different at $P < 0.01$, *t* test. Bars = 50 μm.

Supplemental Fig. S4B), suggesting that *ERF115* and likely its homologs are required cell-autonomously for the replenishment of SSCs upon death induced by DNA DSBs. A similar expression pattern of *ERF115::GUS* was observed in *top1α* roots (Fig. 4C), indicating that *ERF115* and likely its homologs contribute to the continuous replenishment of SSCs in *top1α* roots, which largely sustains their growth. Consistently, we found that overexpression of *ERF115-SRDX*, which could repress endogenous transcriptional activities of *ERF115* and its homologs in wild-type roots and prevent the replenishment of SSCs and the recovery of root growth after the removal of DNA-inducing agents (Heyman et al., 2013), significantly increased the area of cell death in the root meristem of *top1α* (Fig. 4, E–I) and reduced the primary root length of *top1α* (Fig. 4J). Moreover, in the presence of CPT, expression of *ERF115* was similarly activated at the position of SSCs in the wild-type root (Fig. 4D; Supplemental Fig. S4C). Thus, DNA DSBs, induced either by genetic and chemical disruption of TOP1α function or by zeocin, trigger a common response that activates ERF115 and likely its homologs to facilitate the replenishment of SSCs needed for root growth.

TOP1α Is Required to Maintain the Undifferentiated State and Number of CSCs

Within the root meristem, SSCs appeared more sensitive to DNA DSBs than CSCs (Fulcher and Sablowski, 2009). The underlying reasons for this asymmetrical sensitivity within the root stem cell niche have not been elucidated. We noticed that the QC and the root cap meristem became disorganized in *top1α* (Fig. 2B) and therefore used the Lugol's staining method to examine whether there was a change in the undifferentiated state of CSCs. In wild-type roots, the root cap meristem consists of a single layer of starch granule-free CSCs (Fig. 5, A and K), which are maintained in an undifferentiated state and slowly replenished by QC cells (van den Berg et al., 1997; Cruz-Ramírez et al., 2013).

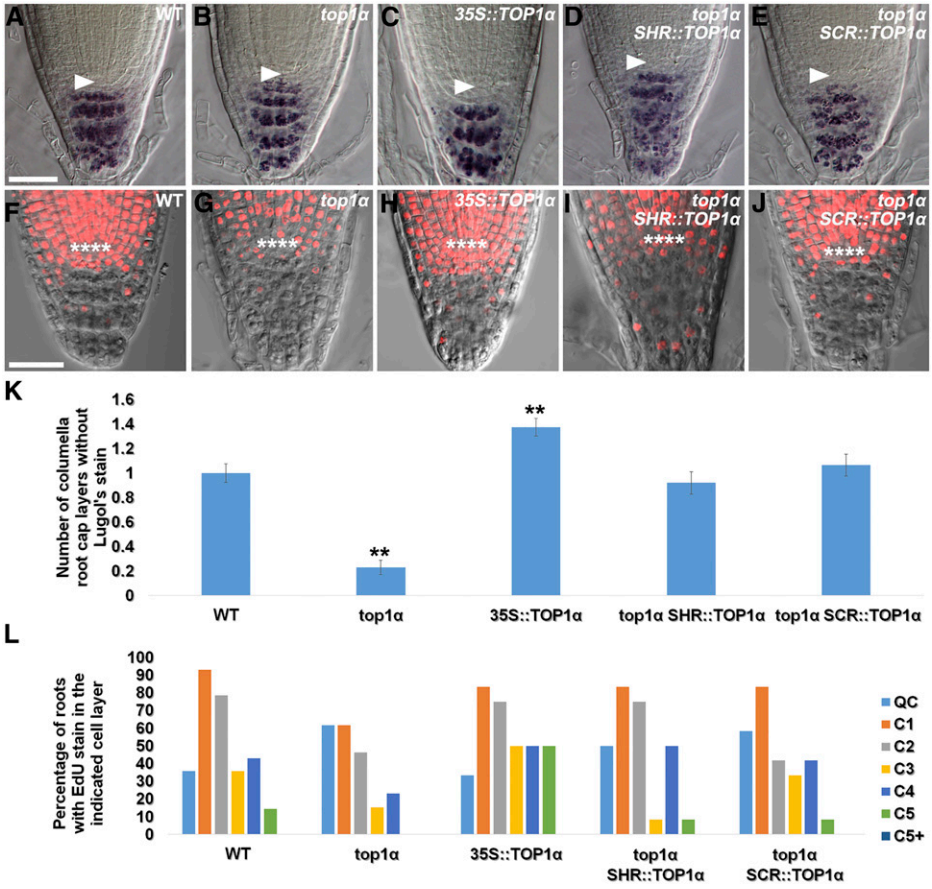

Figure 5. *TOP1α* maintains the undifferentiated state and number of CSCs. A to E, Root tips of wild-type (A), *top1α* (B), 35S::*TOP1α* (C), *SHR::TOP1α* (D), and *SCR::TOP1α* (E) seedlings stained with Lugol's solution. In differentiated columella root cap cells, starch granules with Lugol's stain appear dark blue or purple in color. F to J, Root tips of wild-type (F), *top1α* (G), 35S::*TOP1α* (H), *SHR::TOP1α* (I), and *SCR::TOP1α* (J) seedlings stained with EdU (in red). K, Quantification of the number of columella root cap layers without Lugol's stain. Error bars represent SE ($n > 10$). $^{**}P < 0.01$, *t* test. L, Quantification of the percentage of roots with EdU stain in the indicated cell layer; $n > 10$. Arrowheads in A to E point to the QC, which is marked by asterisks in F to J. Bars = 25 μm.

Each CSC divides asymmetrically to produce two daughter cells: the one immediately distal to the QC retains the undifferentiated state of CSCs, and the other differentiates and accumulates starch granules detectable by Lugol's staining, without undergoing further cell division. In *top1α* seedlings, however, accumulation of starch granules at the position of CSCs was observed (Fig. 5, B and K), suggesting that TOP1α inhibits differentiation of CSCs in wild-type roots. Conversely, *TOP1α* overexpression plants (*35S::TOP1α*) were found to have more layers of starch granule-free CSCs (Fig. 5, C and K), indicating that TOP1α regulates the number of CSCs.

To confirm our results obtained with the Lugol's staining method, we next used 5-ethynyl-2′-deoxyuridine (EdU) to label S-phase CSCs and analyzed the number and distribution of their labeled progeny in wild-type, *top1α*, and *35S::TOP1α* roots after 24 h of labeling (Fig. 5, F–H and L). In wild-type roots, EdU stain could be detected in S-phase CSCs and in their labeled progeny at lower columella layers due to continuous asymmetric division of CSCs and differentiation of lower layer CSC daughters (Fig. 5, F and L). In *top1α* roots, however, reduction of cells with EdU stain were observed at the positions of the CSC layer and lower columella layers (Fig. 5, G and L), suggesting that loss of *TOP1α* function resulted in a loss of undifferentiated state of CSCs. On the contrary, 35S::*TOP1α* had supernumerary EdU-stained cells that accumulated beneath the QC (Fig. 5, H and L), indicating that overexpression of *TOP1α* results in an increase in the number of CSCs.

Moreover, we found that expression of *TOP1α*, either in the stele (*SHR::TOP1α*; Fig. 5, D, I, K, and L) or in the adjacent layer including the QC (*SCR::TOP1α*; Fig. 5, E and J–L), could restore the CSC phenotype (but not the QC phenotype) of *top1α* roots (Fig. 5, B, G, K, and L) to that of wild-type roots (Fig. 5, A, F, K, and L), despite that death of SSCs in *top1α* roots could be prevented by the introduction of *SHR::TOP1α* (Fig. 2E) but not of *SCR::TOP1α* (Fig. 2F). These findings together suggest that (1) death of SSCs in *top1α* roots could cause the CSC phenotype indirectly, likely through affecting the QC signaling required for the maintenance of the undifferentiated state and number of CSCs; and (2) *TOP1α* function in the QC but not in the stele is indispensable for the maintenance of the undifferentiated state and number of CSCs.

TOP1α Maintains the Undifferentiated State and Number of CSCs Downstream of *RBR*

In Arabidopsis, a known key regulator of undifferentiated state and number of CSCs is the single RB homolog RBR (Wildwater et al., 2005). Reduction of

RBR in the QC promotes asymmetric divisions of QC cells that renew CSCs, resulting in supernumerary undifferentiated CSC daughters (Cruz-Ramírez et al., 2013; Fig. 6, A, D, H, and I). Since *TOP1α* overexpression mimics the effect (although weaker; Fig. 5, C, H, K, and L, compared to Fig. 6, A, D, H, and I) of *RBR* reduction on the undifferentiated state and number of CSCs, we next asked whether there is a genetic interaction between *TOP1α* and *RBR* in the maintenance of the undifferentiated state and number of CSCs and generated the following two genetic combinations: *top1α RCH1::RBR RNAi* (*rRBr*) and *35S::TOP1α rRBr*. In *top1α rRBr* roots, increased presence of Lugol's stain and marked changes of cells with EdU stain were observed at the position of QC and CSCs (Fig. 6, B, E, H, and I), as seen in *top1α* (Fig. 5, B, G, K, and L). By contrast, *35S::TOP1α rRBr* had a more extended Lugol's stain-free columella region than either of the parental lines (*35S::TOP1α* and *rRBr*; Fig. 6, C and H, compared to Fig. 5, C and K, and Fig. 6, A and H). The number of EdU-stained cells that accumulated beneath the QC further increased in *35S::TOP1α rRBr* roots (Fig. 6, F and I), compared with that of *35S::TOP1α* (Fig. 5, H and L). However, due to the high number of EdU-stained cells that accumulated beneath the QC, we could not detect a clear difference between *35S::TOP1α rRBr* and *rRBr* (Fig. 6, D, F, and I). Nevertheless, these observations together suggest that (1) loss of *TOP1α* function reverses whereas overexpression of *TOP1α* enhances the CSC phenotype of *RBR* reduction; and (2) *TOP1α* is epistatic to *RBR* in the maintenance of the undifferentiated state and number of CSCs. Consistently, we found that *TOP1α* was up-regulated in *rRBr* roots, whereas the transcript level of *RBR* was not significantly altered in *top1α* roots (Fig. 6G).

DISCUSSION

The integrity of stem cells is of critical importance for the development and growth of multicellular organisms. The sedentary nature of plants means that they must be able to survive various stresses in the soil environment and that maintaining the integrity of root stem cells throughout their entire life span is essential for their ability to withstand stress and sustain growth and productivity (in the case of crop plants). Therefore, it is important to explore and understand molecular and cellular mechanisms that preserve the stem cell pools in plant roots.

Here, we report the discovery of two distinct mechanisms of stem cell maintenance in the Arabidopsis root (Fig. 7). *TOP1α*, but not its only Arabidopsis paralog *TOP1β*, was uncovered as a critical new factor that is required cell-autonomously for survival of SSCs and maintenance of the undifferentiated state and number of CSCs. The lack of observable phenotypes in *top1β* and *RCH1::TOP1β RNAi* indicates that *TOP1α* derives paralog-specific developmental functions after gene duplication, in addition to its redundant (with *TOP1β*), housekeeping role.

In the proximal root meristem, disruption of TOP1α function, either genetically (using the *top1α* mutant) or chemically (by treatment with the TOP1 inhibitor CPT), resulted in preferential death of SSCs (Figs. 2 and 3) due to TOP1-mediated DNA DSBs in S-phase (Fig. 3). Site-directed substitution of evolutionarily conserved amino acid residues critical for the binding of CPT to TOP1 (Sirikantaramas et al., 2008) further revealed that (1) TOP1α is a predominant target of CPT; (2) the TOP1-mediated DNA relaxation activity of TOP1α is essential for the survival of SSCs (Fig. 3); and (3) SSCs are particularly sensitive to torsional stress during DNA

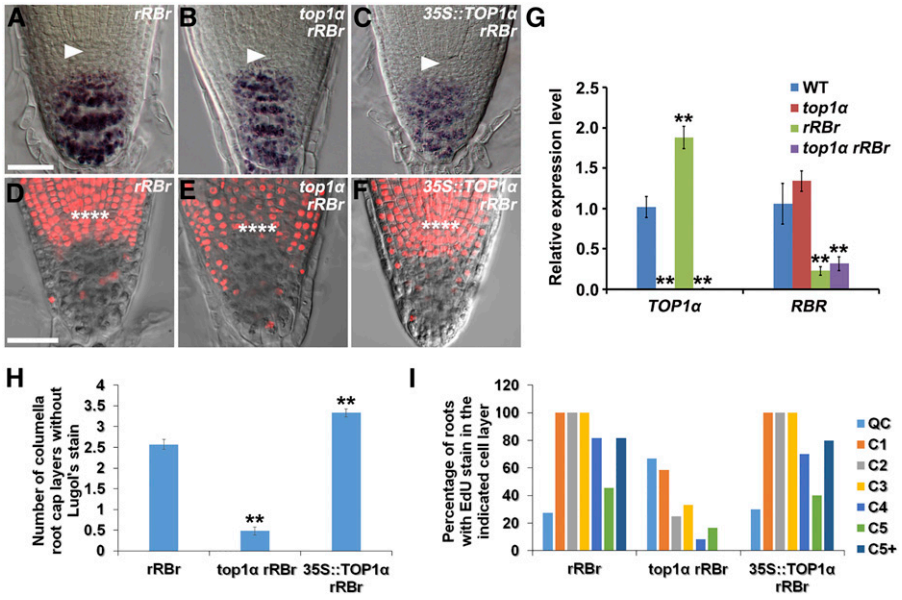

Figure 6. *TOP1α* maintains the undifferentiated state and number of CSCs downstream of *RBR*. A to C, Root tips of *rRBr* (A), *top1α rRBr* (B), and 35S::*TOP1α rRBr* (C) seedlings stained with Lugol's solution. D to F, Root tips of *rRBr* (D), *top1α rRBr* (E), and 35S::*TOP1α rRBr* (F) seedlings stained with EdU (in red). G, qRT-PCR analysis of *TOP1α* and *RBR* transcription in roots of wild-type, *top1α*, *rRBr*, and *top1α rRBr* seedlings. Transcript levels of *TOP1α* and *RBR* in wild-type roots were set to 1. Error bars represent SD from three independent experiments. $^{**}P < 0.01$, *t* test. H, Quantification of the number of columella root cap layers without Lugol's stain. Error bars represent SE ($n > 10$). $^{**}P < 0.01$, *t* test. I, Quantification of the percentage of roots with EdU stain in the indicated cell layer; $n > 10$. Arrowheads in A to C point to the QC, which is marked by asterisks in D to F. Bars = 25 μm.

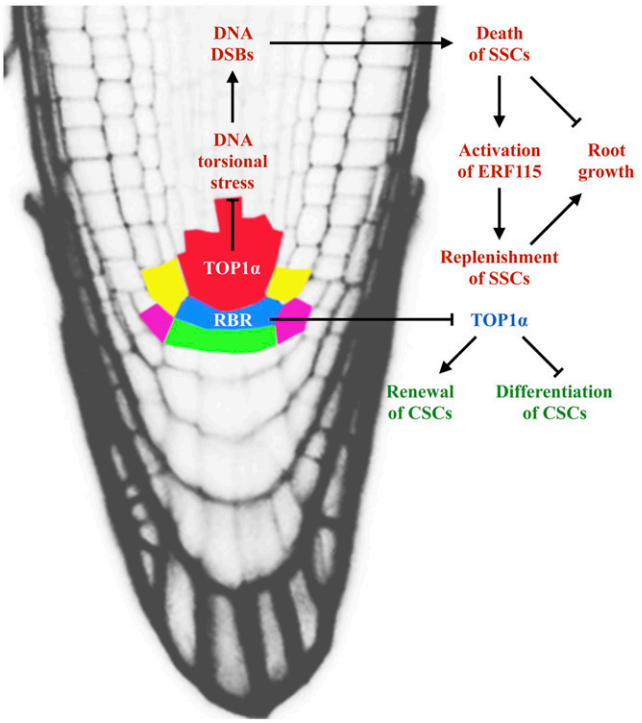

Figure 7. A model for the roles of *TOP1α* in stem cell maintenance in the Arabidopsis root. The diagram illustrates that *TOP1α* acts through two distinct mechanisms to regulate the maintenance of SSCs and CSCs. On the one hand, *TOP1α* is required cell-autonomously for the survival of SSCs. Loss of *TOP1α* function triggers DSBs in S-phase SSCs and results in their death, which can be partially reversed by the replenishment of SSCs mediated by *ERF115*. The integrity of SSCs is essential to ensure continuous root growth. One the other hand, *TOP1a* function in the QC, downstream of *RBR*, is indispensible for the maintenance of the undifferentiated state and number of CSCs.

replication. Notably, several earlier studies have demonstrated that DNA DSB-inducing agents, including replication blocks, could induce death specifically in the SSCs within hours of treatment (Curtis and Hays, 2007, 2011; Fulcher and Sablowski, 2009; Furukawa et al., 2010), suggesting that SSCs are particularly sensitive to DNA DSBs and that TOP1α is one of the key components required for the genome integrity of SSCs that is vital for their survival. Future studies will be needed to reveal why SSCs, compared to other root stem cells such as CSCs, are especially prone to enter a cell death pathway upon detection of DNA DSBs. One hypothesis to be tested is that, as suggested in animals (Loeb and Monnat, 2008), in plants, different stem cell lineages might express different combinations of translesion synthesis DNA polymerases that determine the efficiency of DNA repair (Curtis and Hays, 2007, 2011).

Notably, death of SSCs in *top1α* roots did not cause the loss of the proximal root meristem (Fig. 2), although over the period of analysis *top1α* roots became significantly shorter than wild-type controls (Fig. 1G). By examining the expression of *ERF115* promoter-GUS fusions (Fig. 4; Supplemental Fig. S3), we confirmed our hypothesis that death of SSCs in *top1α* roots was accompanied by continuous replenishment of SSCs, which allowed the maintenance of proximal root meristem and root growth. Consistently, the area of cell death in the root meristem of *top1α ERF115-SRDX* was significantly larger than that of *top1α* (Fig. 4, F, G, and I), and the root length of *top1α ERF115-SRDX* was markedly shorter than that of *top1α* and *ERF115-SRDX* (Fig. 4J), providing persuasive evidence that endogenous transcriptional activities of *ERF115* and its homologs in *top1α* roots are required for the replenishment of SSCs and maintenance of root growth, as previously reported in wild-type roots treated with DNA DSB-inducing agents (Heyman et al., 2013).

In the root cap meristem, differentiation of CSCs was observed in *top1α*, as indicated by the increased presence of Lugol's stain and the reduction of EdU stain (Fig. 5). Notably, death of SSCs, caused by the loss of *TOP1α* function in the stele, appeared to trigger the differentiation of CSCs, but only when expression of *TOP1α* was absent in the QC. These findings led us to conclude that *TOP1α* function in the QC is indispensable for the maintenance of the undifferentiated state and number of CSCs. Terminally differentiated muscle cells in mouse and human were found to be resistant to the effects of DNA DSB-inducing agents (Latella et al., 2004). Similarly, terminal differentiation of CSCs in Arabidopsis may allow these cells to escape from death induced by DNA DSBs, as seen in *top1α* (Fig. 2). More importantly, induction of stem cell differentiation appears to be another general strategy used by animals and plants to maintain the stem cell quality and quantity under genotoxic stress conditions. For instance, DNA DSBs was shown to abrogate or limit self-renewal of mouse melanocyte stem cells and human hematopoietic stem cells by triggering their differentiation (Inomata et al., 2009; Wang et al., 2012).

The retinoblastoma tumor suppressor RB plays a key role in controlling several aspects of stem cell biology. In Arabidopsis, a single RB homolog, RBR, has been identified that can negatively regulate stem cell renewal as observed in animals (Wildwater et al., 2005; Galderisi et al., 2006; Sage, 2012; Desvoyes et al., 2014), suggesting that RB activity is well preserved in evolutionary divergent organisms. Interestingly, our genetic interaction data suggest that *TOP1α* is epistatic to *RBR* in the maintenance of the undifferentiated state and number of CSCs (Fig. 6). RBR appears to be a negative regulator of *TOP1α* (Fig. 6) and acts through repression of *TOP1α* function to regulate the maintenance of the undifferentiated state and number of CSCs (Fig. 6). These findings may help to explain why CPT and another TOP1 inhibitor, topotecan, were found to have potent and fast activity against retinoblastoma, which is caused by the loss of RB function (Chantada et al., 2009; Han and Wei, 2011; Schaiquevich et al., 2014). In the Arabidopsis root, further work is needed to reveal the molecular basis of the genetic interaction, for instance, by analyzing the physical interaction between TOP1α and RBR.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) strains used in this study are of the ecotype Columbia-0. *top1α* (SALK_013164) and *top1β* (SALK_069847) were obtained from the Nottingham Arabidopsis Stock Centre. Primers used for genotyping are listed in Supplemental Table S1. Previously published transgenic lines used in this study include *rRBr* (Wildwater et al., 2005) and *pERF115:GUS* (Heyman et al., 2013). Seedlings were germinated on Murashige and Skoog (MS) agar plates incubated in a near vertical position at 22°C under long-day conditions (16 h of light/8 h of darkness).

Plasmid Construction and Plant Transformation

To generate the *35S::TOP1α* construct for the overexpression of *TOP1α*, the coding sequences of *TOP1α* were PCR amplified, verified by sequencing, and cloned after the *Cauliflower mosaic virus* (CaMV) 35S promoter into the pPLV27 vector through ligation-independent cloning (De Rybel et al., 2011). To generate the *TOP1α::TOP1α*, *SHR::TOP1α*, and *SCR::TOP1α* constructs for the complementation test, the genomic region containing both the coding and 3′-untranslated region sequences of *TOP1α* was PCR amplified, verified by sequencing, and cloned after the CaMV 35S promoter into the pPLV27 vector through ligation-independent cloning. The CaMV 35S promoter was then replaced with the promoter of *TOP1α*, *SHR* (Helariutta et al., 2000), or *SCR* (Di Laurenzio et al., 1996). *TOP1αN871S* was generated with site-directed mutagenesis (Zheng et al., 2004) using the *TOP1α::TOP1α* vector as the template. To generate the *RCH1::TOP1β RNAi* construct, a 3.5-kb promoter region upstream of the *RCH1* (Casamitjana-Martínez et al., 2003) start codon was fused to an inverted hairpin sequence of *TOP1β* as described earlier (Takahashi et al., 2002). All these constructs were introduced into wild-type and *top1α* plants with the floral dip method (Clough and Bent, 1998). To generate *TOP1α::GUS*, *TOP1β::GUS*, and *ERF115::GUS* constructs, 1.3-, 1.1-, and 3-kb promoter regions of *TOP1α*, *TOP1β*, and *ERF115* were fused to a *GUS* reporter gene and nopaline synthase terminator engineered in pGreenII-0229 (www.pgreen.ac.uk), respectively. Each of the resulting constructs was then introduced into wild-type plants with the floral dip method. To generate the *ERF115-SRDX* construct, the coding sequence of *ERF115* was fused upstream of the SRDX sequence and introduced into the pPLV27 vector. The resulting construct was then introduced into wild-type plants via the floral dip method. Primers used for cloning are listed in Supplemental Table S1.

Chemical Treatment

Arabidopsis seedlings were germinated on near-vertically placed MS agar plates for 4 d before treatment. For CPT (Sigma-Aldrich) treatment, 4-d-old seedlings were transferred to fresh MS semisolid medium without (as mock) or with 100 nM CPT for additional 6 or 24 h (unless stated otherwise). For APH (Sigma-Aldrich) treatment, 4-d-old seedlings were transferred to distilled water without (as mock) or with either 48 μM APH or 48 μM APH and 100 nM CPT for indicated time periods. For zeocin (Sigma-Aldrich) treatment, 4-d-old seedlings were transferred to fresh MS semisolid medium without (as mock) or with 13 μM (20 μg/mL) zeocin for 24 h.

Histochemical Analysis of GUS Activity

GUS staining was performed as previously described (Sassi et al., 2012). Samples were incubated in assay buffer at 37°C until sufficient staining was observed. GUS activity was analyzed on a Nikon 80i microscope using Nomarski differential interference contrast optics.

TUNEL Assay for DNA Damage

An in situ cell death detection kit (fluorescein; Roche) was used to perform TUNEL assay according to the manufacturer's protocol with slight modifications. Briefly, the seedlings were fixed in 4% paraformaldehyde in PBS solution for 8 h. After washing, the seedlings were incubated with TUNEL reaction mixture for 1 h at 37°C. The seedlings were washed again and imaged under a Leica TCS SP2 confocal microscope.

Visualization of Live and Dead Cells with PI

PI (10 μg/mL from Sigma-Aldrich, dissolved in water) was used to visualize live and dead cells. Briefly, the roots of the seedlings were submerged in PI on the microscope slide before the coverslip was placed over the root. The roots of the seedlings were then immediately imaged under a Leica TCS SP2 confocal microscope. PI stain is excluded from entering live cells but can penetrate into the dead cells. Quantification of cell death area (μm^2) in the Arabidopsis root was performed as previously reported (Ühlken et al., 2014).

Lugol's Staining

For visualization of starch granules, root tips of 5-d-old seedlings were stained for 1 min in Lugol's solution (Sigma-Aldrich) and then imaged with a Nikon 80i microscope using Nomarski differential interference contrast optics. Quantification of the number of layers of unstained columella root cap cells was performed as previously reported (Hong et al., 2015).

EdU Staining

EdU incorporation assay was performed using a Click-iT EdU Imaging Kit from Invitrogen, according to a previously reported method (Hong et al., 2015). Briefly, seeds were germinated on vertically placed MS agar plates for 3 d. Three-day-old seedlings with uniform root size were then transferred to 10 μM EdU solution and immersed in the solution for 24 h, followed by fixation in 3.7% (v/v) paraformaldehyde (Sigma-Aldrich) for 1 h. Fixed seedlings were incubated with 50 μL Click-iT reaction cocktail for 1 h and imaged with a Leica TCS SP5X confocal microscope. Quantification of the percentage of roots with EdU stain in different columella root cap layers was performed as previously reported (Hong et al., 2015).

qRT-PCR

RNA was isolated from the root tip (<5 mm) with Tranzol reagent (TransGen Biotechnology) according to the manufacturer's protocol. cDNA was prepared with PrimeScript RT reagent kit (RR047A; Takara). Relative expression levels were determined by qRT-PCR with the ABI 7500 real-time PCR system or ViiA 7 real-time PCR system (Life Technologies). *EF1α* was used as reference gene for normalization. Primer sequences used for qRT-PCR analyses are listed in Supplemental Table S1.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *TOP1α*, At5g55300; *TOP1β*, At5g55310; *SHR*, At4g37650; *SCR*, At3g54220; *RCH1*, At5g48940; *RBR*, At3g12280; *ERF115*, At5g07310; and *EF1α*, At5g60390.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *RCH1::TOP1β RNAi*-mediated down-regulation of *TOP1β* had no visible effect on the wild type but caused a rootless phenotype in *top1α*.

Supplemental Figure S2. Expression of wild-type *TOP1α* under the control of its native promoter (*TOP1α::TOP1α*) fully complemented the root growth defect of *top1α*.

Supplemental Figure S3. Zeocin induces preferential death of SSCs in the Arabidopsis root.

Supplemental Figure S4. Similar *ERF115* expression patterns were observed in a previously reported *pERF115:GUS* transgenic line.

Supplemental Table S1. List of primers used in this study.

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