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Report

Dynamic Regulation of Cortical Microtubule Organization through Prefoldin-DELLA Interaction

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Summary

Plant morphogenesis relies on specific patterns of cell division and expansion. It is well established that cortical microtubules influence the direction of cell expansion [1, 2], but less is known about the molecular mechanisms that regulate microtubule arrangement. Here we show that the phytohormones gibberellins (GAs) regulate microtubule orientation through physical interaction between the nuclear-localized DELLA proteins and the prefoldin complex, a cochaperone required for tubulin folding [3]. In the presence of GA, DELLA proteins are degraded, and the prefoldin complex stays in the cytoplasm and is functional. In the absence of GA, the prefoldin complex is localized to the nucleus, which severely compromises α/β-tubulin heterodimer availability, affecting microtubule organization. The physiological relevance of this molecular mechanism was confirmed by the observation that the daily rhythm of plant growth was accompanied by coordinated oscillation of DELLA accumulation, prefoldin subcellular localization, and cortical microtubule reorientation.

Results and Discussion

Hypocotyl elongation is one of the simplest models in which to study plant morphogenesis. Growth of this organ is almost exclusively subtended by anisotropic cell expansion [4], and cell growth direction is determined in part by the orientation of cortical microtubules at the inner tangential wall of epidermal cells, through their influence on the deposition of cell wall material [2, 5, 6]. Despite the observations that suggest that it is possible to uncouple anisotropic growth from the rearrangement of cortical microtubules [7, 8], more recent evidence has confirmed the key role of microtubule organization when this is precisely analyzed in expanding cells within the hypocotyl [1]. Among the signals that regulate microtubule dynamics, the phytohormones gibberellins (GAs) promote cell expansion and also direct the orientation of the cortical microtubule array perpendicular to the growth axis [1, 7, 9], raising the question of how these two processes are coordinated [10].

DELLAs are nuclear proteins that mediate transcriptional regulation of cell expansion genes by GAs [11] and other processes along plant development [12–17]. In *Arabidopsis*, DELLAs are encoded by five genes (*GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3*). They function as transcriptional regulators and, although they lack any known DNA-binding domain, this role is exerted through the interaction with other proteins, in particular, transcription factors [18]. This ability to establish physical interactions also provides the cells with a mechanism for crosstalk between signaling pathways. For instance, the opposite effect of light and GAs on cell expansion is modulated by the interaction between DELLA proteins—whose stability is decreased by the hormone—and PIF (phytochrome-interacting protein) transcription factors—which are destabilized by light [11, 19]. However, this single molecular mechanism does not explain how anisotropic cell expansion is regulated by GAs.

To identify the partners through which DELLA proteins exert their regulatory functions, we performed a yeast two-hybrid screening using a truncated version of GAI that prevents activation of the reporter genes, as shown for RGA, a related DELLA protein [11, 20]. Two of the positive clones encoded full-length versions of prefoldin5 (PFD5), one of the subunits of a heterohexameric molecular cochaperone, conserved from *Archaea* to eukaryotes [21, 22]. Interaction extended also to PFD3, but not to the other members of the PFD complex (Figure 1A; see also Figure S1 available online), indicating that the interaction with GAI was restricted to the α-subunits, which are located at the core of the complex [21]. Coimmunoprecipitation studies in infiltrated *Nicotiana benthamiana* leaves further corroborated this interaction *in planta* (Figure 1B). Given that DELLA proteins are localized in the nucleus [23], whereas the chaperone function of PFD is required in the cytosol [3], we examined the subcellular localization of the GAI-PFD5 interaction by bimolecular fluorescence complementation (BiFC) in *N. benthamiana* leaves [24, 25]. Interestingly, signal from the reconstituted yellow fluorescent protein (YFP) was visible in nuclei of coinfiltrated cells (Figure 1C), suggesting that the interaction with DELLAs might force the accumulation of PFD5 in the nucleus. This idea was further supported by the observation that YFP-PFD5 was detected in the cytosol of *N. benthamiana* leaves (Figure 1D), whereas it mostly appeared in the nucleus when coexpressed with RFP-GAI (Figure 1E).

To confirm that the nuclear accumulation of PFD5 occurs in wild-type *Arabidopsis* plants in a DELLA-dependent manner, we investigated the localization of PFD5 in dark-grown *pfd5* null mutant seedlings complemented with a construct expressing PFD5-GFP under the control of the *PFD5* promoter (see Supplemental Experimental Procedures). As expected, in untreated, control seedlings, PFD5-GFP appeared in the cytosol of cells located in the top third of etiolated hypocotyls (Figure 2A), which correspond to actively expanding, GA-responsive cells [4]. However, incubation with paclobutrazol (PAC), an inhibitor of GA biosynthesis that induces the accumulation of DELLA proteins [23], caused the accumulation of PFD5-GFP in the nucleus, where GFP-RGA accumulates in the control reporter line [26]. Importantly, cytosolic localization was restored 3 hr after application of GA_4 to PAC-grown seedlings, concomitantly with the disappearance of GFP-RGA from nuclei, indicating that PFD5 localization in the nucleus requires the continuous presence of DELLA proteins. Moreover, subcellular fractionation of whole seedling extracts

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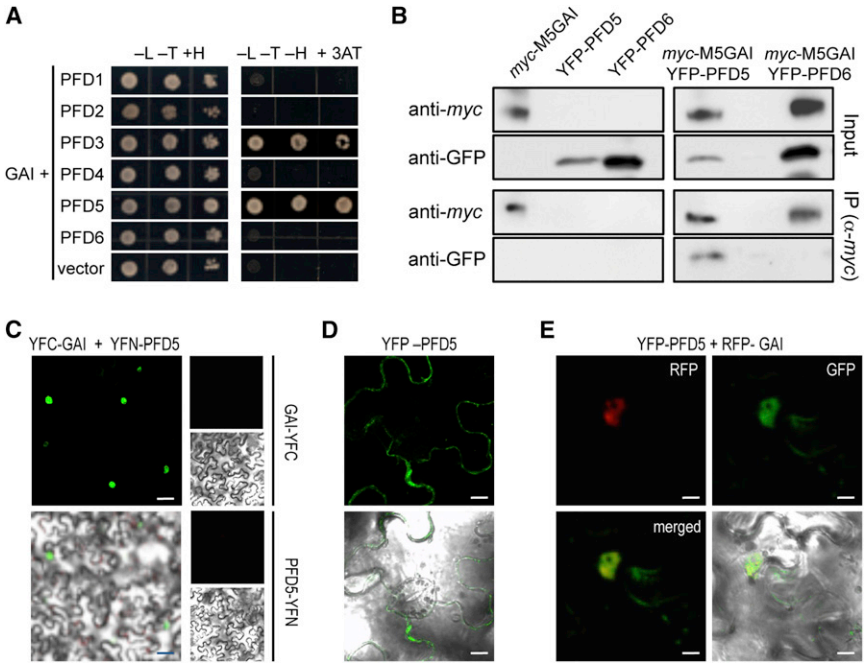

Figure 1. Physical Interaction between GAI and Two Subunits of the Prefoldin Complex in the Nucleus

(A) Interaction between GAI and the different subunits of the *Arabidopsis* PFD complex by a yeast two-hybrid assay. L, Leu; T, Trp; H, His; 3AT, 35 mM 3-aminotriazole. "Vector" corresponds to pDEST22.

(B) Coimmunoprecipitation of *myc*-GAI and YFP-PFD5 expressed in *N. benthamiana* leaves. The "M5" version of *myc*-GAI (see Experimental Procedures) was immunoprecipitated using anti-*myc* conjugated paramagnetic beads, and GAI and PFD5 and PFD6 were detected in immunoblots using anti-*myc* and anti-GFP. Note that PFD6 was not coimmunoprecipitated with GAI. The sizes of the bands correspond to the expected sizes of the fusion proteins.

(C) BiFC analysis of the interaction between GAI and PFD5 in nuclei of *N. benthamiana* leaf cells. Scale bar represents 40 μm.

(D) Localization of YFP-PFD5 in the cytosol of cells of *N. benthamiana* leaves when infiltrated alone. Scale bar represents 20 μm.

(E) Localization of YFP-PFD5 in the nucleus of a representative *N. benthamiana* leaf cell when coinfiltrated with RFP-GAI. Scale bar represents 20 μm.

See also Figure S1.

showed that PFD5 was present in the insoluble nuclear fractions of PAC-treated, wild-type seedlings, whereas it appeared in the cytosolic fraction of seedlings of the pentuple *della* mutant independently of the treatment (Figure 2B). These results indicate that DELLA accumulation is required for the localization of PFD5 in the nucleus.

To assess whether the interaction between GAI and the α-subunits of PFD provokes the accumulation of the whole PFD complex in the nucleus, we examined the subcellular localization of PFD6, a subunit that does not interact directly with GAI (Figures 1A, 1B, and S1). Using a transgenic *Arabidopsis* line expressing *PFD6::PFD6-YFP* (*PFD6-YFP*) [27], we found that PFD6 showed the same behavior as PFD5-GFP (compare Figures S2A and 2A). This result suggests that the integrity of the PFD complex is not altered by the GAI-PFD5 interaction. This is also supported by immunodetection of PFD5 after gel filtration of native extracts of wild-type seedlings grown in the presence of PAC. Under these conditions, PFD5 appeared in the fractions corresponding to the intact PFD complex, similarly to what was observed in the extracts of mock-treated seedlings (Figure S2B). Remarkably, the PFD complex eluted in the same fraction in PAC as in mock, suggesting that DELLA proteins are not permanently associated to PFD or that this interaction is lost during extract manipulation. The idea that PFD localization in the nucleus is dynamic and intimately linked to DELLAs was confirmed using F1 seedlings of a cross between a *GAI::gai-1-GR* line [28], which expresses a dominant version of the DELLA protein that is retained in the cytosol unless the synthetic glucocorticoid dexamethasone (DEX) is supplied, and the *PFD6-YFP* line. As shown in Figure 2C, PFD6-YFP was visible in the cytosol of etiolated hypocotyl epidermal cells, appeared in the nucleus 2 hr after DEX treatment, and was fully nuclear 6 hr after the application of the glucocorticoid. Importantly, the timing for the accumulation of PFD6-YFP in the nucleus coincides with the timing needed by gai-1-GR to exert its transcriptional regulation activity [29, 30], which is consistent with the idea that nuclear accumulation of the entire PFD complex depends upon the presence of DELLAs.

The identification of PFD3 and PFD5 as interactors of DELLA proteins and the relocalization of the cochaperone complex caused by this interaction suggested a mechanism by which GAs could regulate microtubule orientation: the interference with PFD function. In fact, *Arabidopsis* null mutants in at least *PFD3*, *PFD5*, and *PFD6* are defective in PFD function and display a disorganized array of cortical microtubules [22, 27] equivalent to the one caused by GA deficiency (Figure 3A). PFD is required to maintain appropriate levels of tubulin heterodimers in yeast [31], in animals [32], and in plants [22]. The link between PFD activity and microtubule arrangement is based on two pieces of evidence: (1) microtubule growth rate and length are directly proportional to tubulin concentration among other factors [33–35], and (2) cortical microtubule transverse arrangement in hypocotyl cells requires long microtubules [36]. Thus, we hypothesized that the DELLA-dependent nuclear localization of PFD could cause the disarrangement of cortical microtubules by reducing the proper folding of tubulin proteins in the cytosol, similar to the defects found in yeast *gim* mutants affected in PFD activity [37–39]. To test this hypothesis, we analyzed α- and β-tubulin accumulation in their native conformations by gel filtration of extracts of control and PAC-treated seedlings. As expected, most of α-tubulin was recovered in the heterodimer fraction of control extracts, together with β-tubulin (Figure 3B). However, GA deficiency caused by PAC-treatment provoked most of α-tubulin to dissociate from β-tubulin and appear in its monomeric form (Figure 3B). Similarly, a large proportion of β-tubulin shifted from tubulin heterodimers to a lower-size fraction that could represent complexes with unidentified proteins. In summary, our results suggest that DELLAs indirectly affect the polymerization of microtubules (and ultimately their ability to reorient) by controlling the availability of α/β-tubulin heterodimers through, at least, the prevention of PFD function as a tubulin cochaperone in the cytosol.

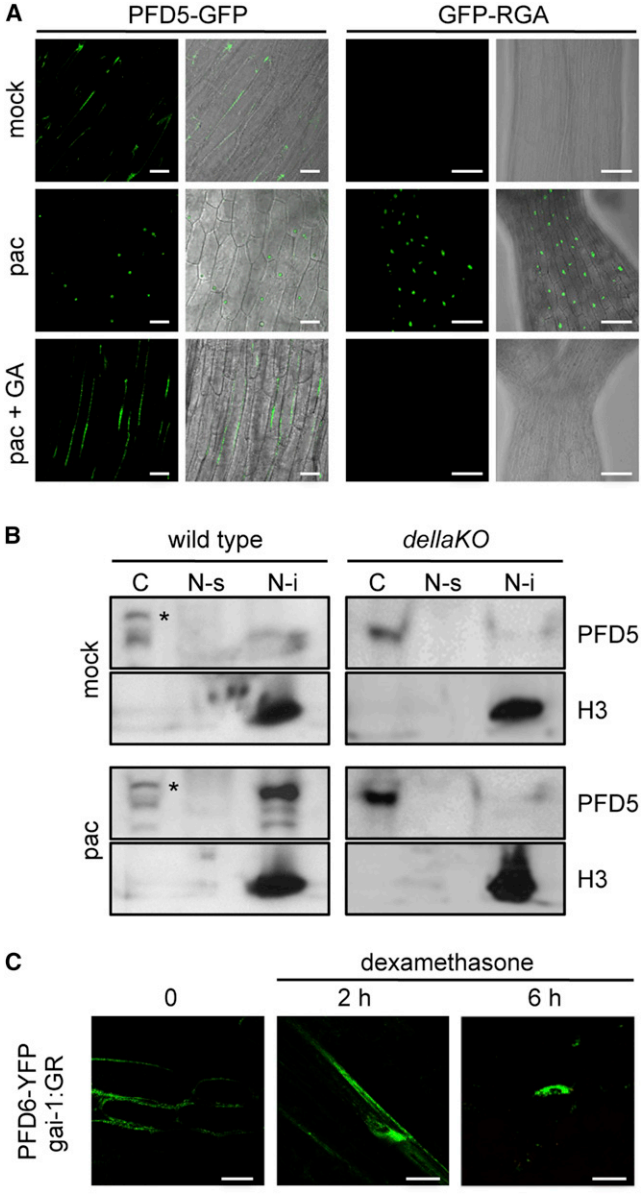

Figure 2. DELLA-Dependent Nuclear Accumulation of PFD in *Arabidopsis* Hypocotyls

(A) Confocal images of PFD5-GFP and GFP-RGA in the apical third of wild-type *Arabidopsis* hypocotyls grown for 3 days in darkness in MS medium (mock) or in MS supplemented with 1 μM PAC ("pac" in figure) and 3 hr after the application of 10 μM GA_4 to PAC-grown seedlings (pac + GA). Scale bar represents 40 μm.

(B) Immunochemical detection of PFD5 and histone H3 proteins in fractionated extracts of *Arabidopsis* seedlings. Histone H3 was used as a marker for the insoluble part of the nuclear fraction. C, cytosol; N-s, soluble fraction of the nucleus; N-i, insoluble fraction of the nucleus. The sizes of the bands correspond to the expected sizes of the corresponding proteins (15 kDa for PFD5, and 15–17 kDa for H3). The asterisk marks the band with the predicted size when multiple bands are visible.

(C) Fluorescence of PFD6-YFP in a F1 seedling from a cross with a *GAI::gai-1-GR* line, before and 2 hr and 4 hr after the application of 10 μM dexamethasone. Scale bar represents 10 μm.

See also Figure S2.

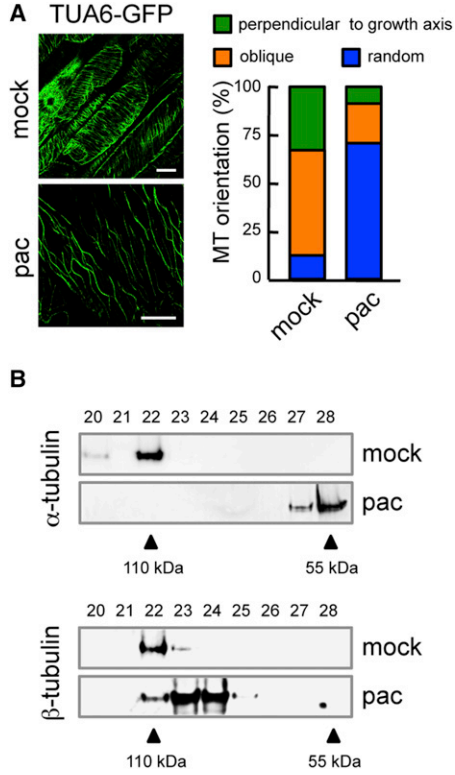

Figure 3. DELLA-Dependent Regulation of Cortical Microtubule Arrangement and α/β-Tubulin Heterodimers Availability

(A) Confocal images of cortical microtubules labeled with GFP in the epidermal cells of the top third of hypocotyls of a *TUA6-GFP* line. Scale bar represents 10 μm. Quantification of the orientation of cortical microtubules was carried out in at least 120 individual cells.

(B) Immunodetection of α- and β-tubulin in size-fractionated protein extracts after gel chromatography. Figures indicate the fraction number.

In all panels, seedlings were grown for 3 days in darkness in MS (mock) or in MS supplemented with 1 μM PAC ("pac" in figure).

The growth rate of *Arabidopsis* hypocotyls oscillates in a daily fashion, with maximal growth occurring at the end of the night [40, 41]. The regulation of DELLA protein stability by the circadian clock is key for this oscillatory behavior [42]. Therefore, if DELLAs coordinately regulate both expansion and growth direction, an important prediction is that the subcellular localization of PFD and the predominant orientation of cortical microtubules should be dictated by the phase of DELLA accumulation under photoperiodic conditions. Indeed, we found that microtubules in hypocotyl epidermal cells displayed a preferred orientation perpendicular to the cell expansion axis during the growth phase toward the end of the night (ZT19) and random or longitudinal shortly after dusk (ZT9) (Figures 4A and 4B), coinciding with the slowest growth rate. This indicates that the orientation of the cortical array of microtubules is dynamic and subjected to diurnal regulation. More importantly, disorganized microtubule arrangement correlated with the accumulation of GFP-RGA at ZT9 and the nuclear accumulation of PFD6-YFP, whereas the transverse organization coincided with the presence of PFD6-YFP in the cytosol (ZT19) and undetectable GFP-RGA levels (Figure 4A). As expected, nuclear PFD6-YFP accumulation at ZT9 was not visible in seedlings continuously grown in the presence of GAs (Figure S3).

To gauge to what extent the DELLA-dependent daily changes in PFD localization affects the function of the complex, we examined other PFD-regulated processes, for instance the expression of tubulin genes, reported to be under

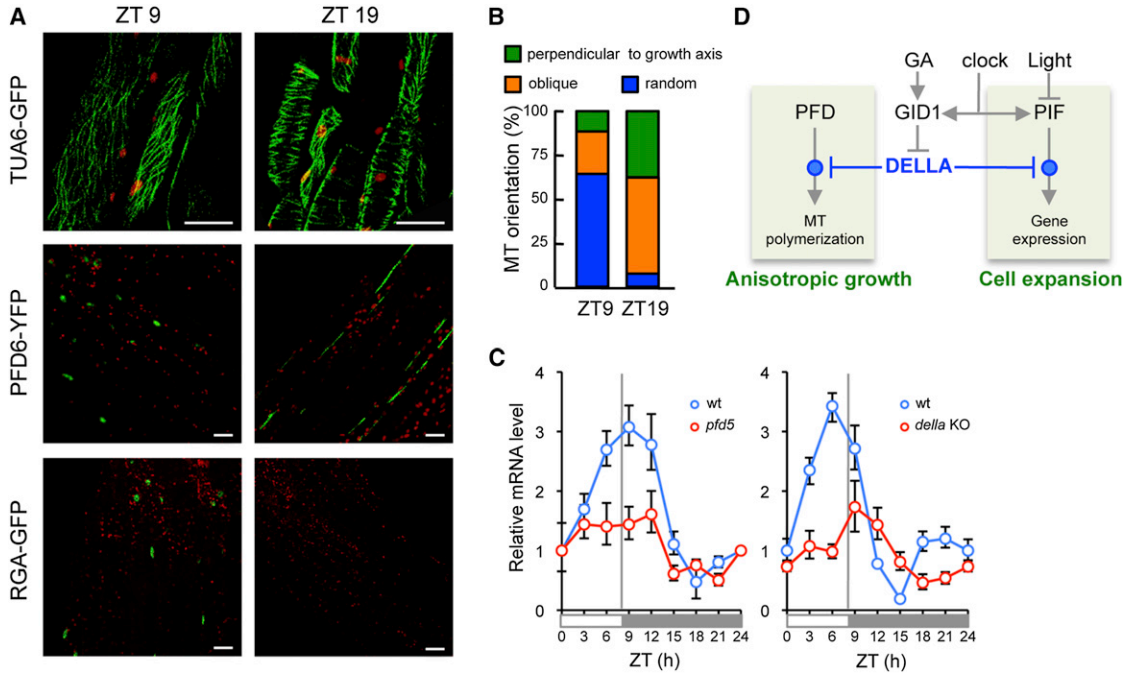

Figure 4. Temporal Coordination of PFD Accumulation in the Nucleus and Cortical Microtubule Arrangement

(A) Confocal images of TUA6-GFP, PFD6-YFP, and GFP-RGA in the epidermal cells of the top third of hypocotyls of seedlings grown under short days (8 hr of light and 16 hr of darkness). ZT, time (in hours) after lights on. Scale bar represents 20 μm.

(B) Quantification of the orientation of cortical microtubules, which was carried out in at least 115 individual cells.

(C) Oscillation of *TUA2,4,6* expression in seedlings grown under short-day cycles in wild-type (Col-0) and *pfd5* mutant seedlings (left) and in wild-type (L*er*) and pentuple *della* mutant seedlings (right). White and gray bars represent day and night, respectively. Data points represent the mean ± SEM of three biological replicates. The primers used in the RT-qPCR experiment do not distinguish between the three *TUA* genes, due to the high identity percentage of their nucleotide sequence [43].

(D) Model of the coordination by DELLAs of the three aspects that conform organ growth by cell expansion: temporal control of growth through the oscillation of DELLA stability, control of cell expansion through physical interaction between DELLAs and PIF (and possibly other) transcription factors, and determination of growth direction through physical interaction between DELLAs and PFD with predictable impact on the polymerization of cortical microtubules.

See also Figure S3.

the control of PFD in yeast [38]. Given that the expression of several *TUA* genes (encoding α-tubulin) in *Arabidopsis* oscillates in a daily fashion, according to the DIURNAL database [44], we examined the effect of *pfd5* and pentuple *della* mutations in the cyclic behavior of these genes. Consistent with the observation in yeast, expression of α-tubulin genes required the activity of PFD5 (Figure 4C) and, more interestingly, their daily oscillation was severely affected in a pentuple *della* mutant as well, supporting the idea that DELLA-dependent accumulation of PFD in the nucleus is required for the expression of several *TUA* genes. Interestingly, maximal daily expression of the examined *TUA* genes did not coincide with the period of maximal growth rate, in agreement with the idea that translational regulation and folding of tubulin is a critical step.

Our results underscore the role of DELLA proteins as coordinators of distinct processes required for plant growth and provide a molecular link between the timing, the execution, and the direction of cell expansion (Figure 4D). The alteration in PFD function through protein-protein interaction has also been described in animal cells during viral infection [45] and may represent a strategy for the control of tubulin availability. However, although a proper tubulin concentration is a prerequisite for the orientation of cortical microtubules, additional mechanisms have to be invoked to determine the final organization of the microtubule arrays during cell expansion [46–48]. The DELLA-dependent localization of PFD in the nucleus is at the core of this mechanism. It impairs the activity of PFD as a chaperone in the cytosol, but our results also raise the intriguing possibility that PFD may have an additional role in the nucleus. Nuclear localization of PFD5 has been described in animals, where it acts as a regulator of the c-Myc transcription factor [49–51]. It is therefore possible that the PFD complex or its subunits perform a similar, DELLA-dependent role in regulating transcription in plant cells.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.03.053.

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