Investigating the apical notch, apical dominance and meristem re-emergence in *Marchantia polymorpha*.

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Abstract

Meristems are the growth centres of plants, and fundamental in understanding plant development, morphogenesis and vegetative propagation. Across all plant groups, the phytohormone auxin controls meristem maintenance, represses the emergence of new meristems (apical dominance) and mediates cellular reprogramming when new meristems regenerate following damage to existing meristems. The liverwort Marchantia produces clonal propagules (gemmae) featuring two meristematic apical notches, presenting a tractable experimental system to study meristem biology. I used laser ablation microscopy to precisely disrupt these apical notches, finding that the first apical cell layer is indispensable for meristem maintenance. Within this layer, a contiguous quorum of cells is required for meristem activity, depending on the status of surrounding meristems. Apical notches reorientate in response to damage, demonstrating that the apical cells act as a communicating population. Unlike the current model, where a single Apical Cell at the apex (centre) of the notch produces and is surrounded by subapical cells, this updated model shows that feedback from the apical cell population is necessary to maintain meristem activity and to define the apex of the notch. These experiments show communication between notches and regenerating meristems, with heterogeneity across the gemma tissue for carrying these signals. The apical dominance signal represses cell division and requires both sources and sinks, features of auxin-mediated communication. I present a model of Marchantia meristem organization, involving intra-, inter- and extra-meristem communication. This provides a framework for further study of meristem communication, formation and maintenance in Marchantia and improving knowledge of plant meristems more widely.

Significance Statement

Meristems are the growth centres of plants. Understanding how meristems function, how existing meristems stop new meristems emerging (apical dominance) and how cellular reprogramming regenerates meristems is central to understanding plant development. The liverwort Marchantia is an increasingly important model plant, as it offers a streamlined system whose evolutionary position makes its biology relevant to all plant groups. Marchantia apical notch meristems display regenerative and dominance behaviour typical of plant meristems. I used laser ablation to make precise disruptions to the apical notch, producing an improved model of Marchantia meristem organization involving intra- and inter-meristem communication characteristic of the hormone auxin. This will inform future Marchantia research and provide a simplified experimental framework for the study of complex meristematic processes.

Main Text

Introduction

Cell division in plants occurs in meristematic regions. Meristems contain pluripotent stem cells which continually divide asymmetrically to produce a daughter and a meristematic cell. The

cells at the centre of the meristem divide slowly, while those at the periphery divide faster. This moves daughter cells outwards as they mature and differentiate, while meristematic cells remain in the centre, replenishing the stem cell pool. This spatial distinction between division and differentiation allows plant meristems to sustain growth and new organ formation simultaneously (1). It means that meristem size and spacing must be controlled for normal morphogenesis. All plant groups do this by means of positive and negative feedback loops involving signalling molecules such as CLE peptides and phytohormones. Disrupting the biosynthesis or transport of these signalling molecules leads to oversized meristems, incorrectly spaced meristems, or loss of meristem function (2, 3).

Perhaps the most important of these signalling molecules is the phytohormone auxin. In vascular plants, auxin is produced in the shoot apical meristem (SAM) (4) and flows outwards by polar transport, where it is involved in controlling cell expansion and differentiation (5, 6). Secondary meristem formation is induced by the formation of local auxin maxima and minima arising out of auxin gradients extending from the apical meristems (6). Apically derived auxin inhibits growth of secondary meristems in the buds: this is apical dominance (3). If the SAM is damaged or destroyed, the flow of auxin is disrupted, apical dominance is lifted and these meristems are released from dormancy and begin growing (7). Plants have the capacity to regenerate meristems in response to localized wounding, which changes the auxin:cytokinin ratio around the damage site. In vascular plants, this causes cells from certain tissue types (e.g., embryonic tissue, pericycle) to lose their previous identity, respecify their cell fate and become meristematic tissue (6, 8). This cellular reprogramming to acquire meristem identity involves changes in gene expression, such as the activation of WIND, WUS, WOX and PLT transcription factors (8). Bryophytes, such as the liverwort *Marchantia polymorpha*, have massive capacity for regeneration: all parts of the plant, and even single cells, can regenerate meristems *de novo* that eventually produce full plants (9–14).

Marchantia grows as a dominant haploid gametophyte thallus, from which emerges a diploid sporophyte. The sporophyte lacks an apical meristem, instead growing by cell division along its length (15). The gametophyte thallus has apical meristems located in concave apical notches (Fig. 1*A*), which are the loci of cell proliferation. The apical notch consists of a central Apical Cell with four cutting faces, surrounded by dorsal and ventral merophytes, plus lateral subapical cells (16, 17). Around each apical notch is a region of cell differentiation. As cells move outwards away from the meristem, division rates reduce, and cell expansion increases. The apical meristems appear early in sporeling development, and their emergence marks the transition from an undifferentiated callus-like protonema into a prothallus, which goes on to form the thallus (10, 18). As the thallus grows the apical notch meristems enlarge and bifurcate, thus producing new thallus branches by dichotomous branching (19). The thallus also forms dorsal outgrowths called gemma cups, within which grow vegetative propagules called gemmae (20). Gemmae have two apical notch meristems located at either end (Fig. 1*B*). Gemma meristems remain dormant until the gemma is removed from the gemma cup, at which point they germinate and grow a new thallus.

Marchantia apical notch meristems demonstrate apical dominance dynamics and lateral inhibition (21). Apical notches prevent the emergence of new active meristems (outside of dichotomous branching) and repress dormant meristems, e.g., gemmae within the gemma cup (22), shaded apical notches (19). If active apical notches are removed, then meristems kept dormant by lateral inhibition start growing (23). If all apical notch meristems are removed then apical dominance is lifted and meristems regenerate *de novo* (9, 12). Auxin has long been known to be involved in meristem regeneration in Marchantia (21, 23–26). Auxin is produced in the apical notch and is exported to the rest of the thallus, where it inhibits meristem formation (i.e., apical dominance). Removing apical notches removes these auxin sources. Initially auxin levels drop in the remaining plant, and then rise as new meristems emerge (9).

Developing a better insight into how meristems function is key to understanding plant development and producing beneficial new morphologies (2, 3). Understanding the molecular mechanisms of meristem regeneration and cellular reprogramming to convert different cell types into meristematic tissue is important in vegetative propagation of agricultural crops and offers an

avenue to develop new transgenic methods (8, 27, 28). Considerable progress on understanding apical meristems has been made from study of angiosperms, particularly *Arabidopsis* (29), however these systems have limitations, such as difficult access for imaging (particularly live imaging) (30, 31) and the complexity of the gene networks involved (2, 5).

Although the Marchantia apical notch has received relatively little attention compared to angiosperm meristems (32, 33) it offers a promising opportunity to gain insights into plant meristems generally. The key advantage of Marchantia as an experimental system for plant biology is its streamlined nature. For many gene families the Marchantia genome has fewer copies compared to other model plants like Arabidopsis or Physcomitrella (32, 34), notably the minimal set of components related to auxin biosynthesis, transport and signalling (34–39). It is highly experimentally tractable, with fast growth rates and well-established transgenic protocols (40, 41). The gemma is well-suited for developmental experiments, since it grows in a stereotypical pattern. Crucially, gemmae have open development where almost all of the cells and tissues are easily accessible for microscopy without the need for dissection, sectioning or clearing (40, 42).

Marchantia, as a bryophyte, occupies an important position in the land plant phylogeny (43–46). Although the thalloid gametophyte is not homologous to the dominant diploid sporophyte of vascular plants, phylogenetic analyses and molecular research finds that the apical notch meristem shares many regulatory modules in common with angiosperm apical meristems (47–53). The SAM model is thought to be how meristems operated in the first land plants (54), making findings from Marchantia applicable to all plant meristems (55). The two apical notches in the Marchantia gemma allows study of inter- and intra-meristem signalling, and of the gene regulatory networks influencing meristem morphology (9, 47, 48, 56). Meristem regeneration in gemmae provides an opportunity to investigate cellular reprogramming and how emerging meristems co-ordinate. This can provide insights into how Marchantia specifies new meristems during spore germination (10, 18), gemma cup development (20, 57) and apical notch bifurcation (19, 47), processes relevant to vegetative reproduction and lateral branching in crop species. In addition to its importance in plant evo-devo studies, Marchantia holds great promise as a chassis for synthetic biology (40, 58), therefore greater understanding of Marchantia meristems will inform this research.

I used laser ablation and transgenic marker lines to make precise disruptions in gemmae meristems and observed how this altered meristem-related processes (59–61). My results point towards a model with a dynamic population of apical cells communicating within the apical notch and with the rest of the gemma, and requiring a balance of auxin sources and sinks to maintain meristem activity. Under this model, disruption of this communication and balance induces new meristems to emerge, or existing meristems to become inactive and lose their identity.

Results

Laser ablation of the Apical Notch induces meristem regeneration

Marchantia plants expressing a plasma membrane marker fused to the red fluorescent protein mScarlet allowed the visualization of new cell membranes and a way to monitor cell division. Laser ablation microscopy was used to remove both notches of a 0 days post germination (dpg) gemma (Fig. 1*C*) and the membrane marker line used to track the regeneration process (Fig. 1*D*). This confirmed previous findings (62), that there are four distinct phases of regeneration in Marchantia. In the first ~24 hours cell division remains largely dormant. Between 24-48 hours after ablation there is rapid, widespread cell division across the gemma. After ~48 hours cell division becomes restricted to localized patches of cell division (dashed circles). Approximately 5 days after ablation (DaA) meristem regeneration has been completed (yellow arrows), forming full apical notches.

A collection of enhancer trap lines where a nuclear mVenus signal marks the apical notch/meristem region (10) were used to monitor meristem activity and regeneration. The location and progress of meristem regeneration could be easily monitored using the enhancer trap signal (SI Appendix Fig. S1). Nuclear mVenus marks patches of localized cell division and only persists

in actively dividing regions that produce full meristematic apical notches. Loss of marker signal means that an apical notch has stopped cell division activity and lost meristem identity.

Examination of multiple gemmae with both notches completely removed (ablation pattern in Fig. 1*C*) did not find any clear predictability as to which regions would go on to form new meristem, nor how many patches of localized cell division or fully regenerated meristem there would be. The exception to this is the stalk scar region, which was never observed to initiate cell divisions or regenerate meristems.

Laser ablation microscopy can make fine scale perturbations in the apical notch to see how much tissue must be removed to induce meristem regeneration. One apical notch was entirely excised and in the other notch a number of rows of apical notch cells were ablated. Ablating one cell row deep into the apical notch was sufficient to induce meristem regeneration (Fig. 1*E*, *F* and SI Appendix Fig. S2*A*, *B*). Notch organisation was disrupted following ablation, with the area expanding outwards and cell divisions (and eventually meristem regeneration) always occurring in the region behind the ablated row of cells (yellow arrow, Fig. 1*F,* SI Appendix Fig. S2*B*). Ablating the first three rows of cells was required for meristem regeneration to be induced elsewhere in the gemma, away from the notch (SI Appendix Fig. S2*C*, *D*).

Laser ablation microscopy demonstrates cellular interactions within and between apical notches

The current Marchantia literature centres around a model where the key feature of each apical notch meristem is a single Apical Cell. This divides, giving rise to subapical cells, which comprise the rest of the first row of cells in the apical notch. However there has been little experimental detail on how these cell types are specified, how they interact within each other or with other apical notches (16, 17, 32). I therefore interrogated this model in more detail by ablating specific parts of the crucial first row of apical notch cells. A flow chart of the outcomes of these experiments is provided in SI Appendix Fig. S3.

Ablating one side of the apical notch did not disrupt meristem function, irrespective of whether or not there was another intact apical notch present in that gemma (SI Appendix Fig. S4*A*, *B*). However, leaving only a few apical notch cells intact on one side disrupted meristem function. If the other apical notch in the gemma was intact (SI Appendix Fig. S4*C*) then the partially ablated notch lost meristem identity due to apical dominance from the intact notch. If the other notch was excised (SI Appendix Fig. S4*D*), the remaining cells in the partially ablated notch were insufficient to sustain apical dominance, and new meristematic regions emerged elsewhere in the gemma fragment. When a few apical notch cells were left intact on both sides of the notch then this partially ablated notch always lost meristem identity (SI Appendix Fig. S4*E*, *F*). Notches with multiple small subpopulations of intact cells lost meristem identity, even if there were more intact cells in total compared to functional notches where the intact cells comprised a contiguous subpopulation.

When both sides of the apical notch were ablated, but a few cells at the apex (including the presumed Apical Cell) were left intact, then the outcome depended on the status of the other notch in the gemma. If the other notch was excised (Fig. 2*A*), then the intact apex continued to function as a meristematic apical notch (blue arrow) and retained apical dominance as no new meristematic regions emerged elsewhere. When the other notch in the gemma was intact (Fig. 2*B*), then the intact apex alone could not sustain meristem function and became inactive (grey arrow), even if more cells remained compared to cases where meristem function continued with the other notch excised (e.g., Fig. 2*C*).

Parts of the notch spanning the apex and sides were ablated, including the central region of the notch where, according to the traditional model, the Apical Cell is located (Fig. 2*D*, *E*). When some of the apical notch cells were ablated, but a sufficient contiguous subpopulation of apical notch cells remained, then not only did the notch continue meristematic activity (blue arrows), but over the next 48 hours its growth pattern changed. By 3DaA, what was previously the centre of the intact subpopulation of apical notch cells has become the centre of the apical notch, i.e., the notch had reorientated. This occurred irrespective of where the centre of the original notch had been before ablation, and did not appear to be influenced by whether the

original centre of the notch (i.e., the location of the Apical Cell) was ablated or not. During the first 48 hours after ablation, no new regions of cell division appeared (if the other apical notch in the gemma had been removed), nor were reorientating notches subject to apical dominance-related inhibition (if the other notch in the gemma was intact). This indicates that reorientating notches continued to be functional, apically dominant meristems regardless of whether there was another intact apical notch in that gemma. This means that the morphological changes involved in notch reorientation were not due to meristem regeneration.

Meristem regeneration involves communication between regions of cell division

Fig. 3 and SI Appendix Fig. S5 show a gemma ablated so that one apical notch was entirely excised and in the other notch a short, contiguous subpopulation of apical cells was left intact. Initially, the rest of the gemma was released from apical dominance and proceeded to the early phases of regeneration. By 2DaA there were regenerating patches of localized cell division with dense enhancer trap marker signal (dashed circles, Fig 3*C*). Simultaneously, the partially ablated apical notch also recommenced cell division (white arrow, Fig. 3*C*). By 3DaA this region was the most prominent area of marker signal, though other patches of signal were visible (SI Appendix Fig. S5*D*). Five days after ablation (Fig. 3*D*, SI Appendix Fig. S5*F*), the notch that was originally partially ablated had become a fully active, mature notch (blue arrow). It had re-established apical dominance, and the other patches stopped division and meristem regeneration (indicated by loss of marker signal, green arrows). However, these areas progressed considerably along the meristem regeneration pathway, with sufficient cell division to produce protrusions on the thallus.

Directional flow of signal outwards from the apical cells

The apical dominance signal produced at the apical notch must flow outwards in order to influence the rest of the gemma. Tissue was ablated at different distances and in different orientations to the apical notch to see if the resulting incisions could interrupt the flow of signal. Incisions made perpendicular to the notch had no obvious effect (SI Appendix Fig. S6*A*-*E*). Incisions made parallel to the notch (SI Appendix Fig. S6*F*-*O*) resulted in more cell division proximal to the incision and increased cell expansion distal to the incision. This effect was not seen when the apical cells proximal to the parallel incision were ablated; instead the cells on both sides of the incision ceased dividing and showed increased cell expansion.

More extensive incisions were made to test the limits of apical dominance signal transport (Fig. 4). This found that the type of connection to the rest of the gemma was crucial. Connections through tissue from the central part of the gemma (Fig. 4*A*-*C*) communicated the repressive signal from the active meristematic apical notch (Fig. 4, blue arrows) and did not see meristem regeneration elsewhere. This was the case even when the connecting tissue was only a few cells wide.

In contrast, connections formed from tissue around the gemma periphery (Fig. 4*D*-*F*) did not communicate the repressive apical dominance signal. Multiple new meristematic regions emerged in the rest of the gemma, despite there being a fully functioning meristematic apical notch present. If the region around the original apical notch included significant amounts of surrounding tissue (Fig. 4*E,F*), then the apical notch continued to grow and develop as normal (blue arrows), even as new meristematic regions emerged elsewhere (dashed circles).

If this region contained the apical notch and cells immediately surrounding the notch, then the outcome was different (Fig. 4*G, H*). The rest of the gemma was released from apical dominance and proceeded through the early phases of meristem regeneration (dashed circles). The original notch sustained meristem identity for the first day after ablation, as shown by enhancer trap marker signal persisting. At 2DaA the marker signal faded and cell division ceased, and by 3DaA the notch had become inactive (grey arrows). The cells did not divide and instead expanded, and no marker signal was present. This was the same as cases where the apical

notch and immediately surrounding region was totally disconnected from the rest of the gemma by ablation of the intermediary tissue (SI Appendix Fig. S7).

This observed self-inhibitory feedback is characteristic of auxin (35, 63). A reporter line marking expression of the canonical polar auxin transporter MpPIN1 (36, 46) shows that in 0dpg gemmae expression is focused around the apical notches, and is present around the gemma edge and in occasional cells across the centre of the gemma. This suggests that the apical notch meristem is an auxin source, but that auxin signalling is more widespread. MpPIN1 marker line gemmae were ablated according to the incision patterns described above (see Fig. 4*C, F, I*). Fig. 4*C* showed no MpPIN1 expression central tissue connection, yet the intact notch retained apical dominance over the rest of the gemma and no new meristems emerged. Conversely, Fig. 4*F*, *I* show gemmae ablated such that the intact notch was connected to the rest of the gemma by peripheral tissue. The peripheral connections displayed MpPIN1 expression, yet meristem regeneration occurred elsewhere in the gemma (dashed circles). The reduced MpPIN1 expression in the notch by 3DaA in the gemma in Fig. 4*I* indicated loss of meristem activity (grey arrow).

Larger gemma fragments regenerate meristems faster

Since the size of the region surrounding the notch has an effect on meristem maintenance or self-inhibition, I also examined how the size of the gemma fragment influences meristem regeneration. A standard-sized area around the apical notch region was entirely excised in a number of gemmae from the enhancer trap lines ET239-P125 and ET239-P33. The remaining gemma fragments were then ablated to leave a a circle of tissue with a diameter 40, 60, 80 or 100% the notch-to-notch distance of the original gemma. The reappearance of the enhancer trap marker signal and the appearance of notch morphology was used to measure the speed of meristem regeneration (Fig. 5, see SI Appendix Fig. S8 for a representative set of images from the time course). The timings of when dense patches of marker signal appeared (dashed circles, SI Appendix Fig. S8), and when a meristem with notch-type morphology re-formed (yellow arrows, SI Appendix Fig. S8) were recorded (Fig. 5). Over the 108-hour time course it was observed that larger fragments had patches of marker signal reappear earlier and had faster regeneration of full meristems with apical notch morphology compared to smaller fragments.

Discussion

Laser ablation of transgenic Marchantia lines reveals minimum requirements for meristem regeneration

Previous experiments investigating Marchantia regeneration were limited to relatively crude surgical manipulations using cutting with scalpel blades (9, 12, 13, 23, 64). This work focused on older thalli, since young gemmae were too small to manipulate, and involved removing all of the apical notch. These cutting experiments demonstrated that when all meristematic apical notches were removed from a thallus, then new meristems would regenerate, producing new apical notches. If one apical notch was intact then no new meristems would emerge.

Laser ablation microscopy allows for fine scale disruptions and precise removal of cells in the apical notch to determine the minimum amount that needs to be removed to induce meristem regeneration. This demonstrated that the first row of apical cells is indispensable for meristem maintenance. It also reveals a previously unappreciated substructure within the apical notch region, with the immediate first row of apical notch cells acting as meristematic organizers and the second and third rows being “primed” for meristem regeneration in preference to the rest of the gemma. I infer that because the cells in the second and third rows of the apical notch are divisionally active, their expressed gene regulatory networks are still those of early ontogeny (33).

This gives them a “head start” over other parts of the gemma as the cells require less reprogramming to acquire meristem identity.

Beyond this there appears to be no wider predictability or clear bias as to which regions will regenerate new meristems (13). This is different to many vascular plants, where in the post-embryonic plant only certain somatic cell types are competent for new meristem formation. This regenerative flexibility was reported in early studies (13), but is in contrast to surgical cuts on older thalli, where regeneration always occurred at the apical midrib on the basal explant (9, 12). The exception to this is the stalk scar region which was never observed to regenerate new meristem. Stalk scar cells are the oldest cells in the gemma, produced at the initiation of gemma formation within the gemma cup (16, 20). It could be the case that, just as the youngest cells in the gemma are primed for meristem regeneration, the oldest cells are excessively complex to reprogram to become divisionally active.

Apical Notch Meristem Organization and Communication

The standard model of the Marchantia meristem focuses on the Apical Cell, a single pluripotent cell with four division planes, located at the centre (apex) of the notch. The Apical Cell divides to form dorsal, ventral and lateral merophytes, which then go on to form the various tissues of the thallus (16, 17, 32). Under this model the meristem is defined by the presence of an Apical Cell, since it is the only cell type not produced by any other cells in the notch. Bryophytes are taken to have a single apical cell (33), whereas in more complex plant meristems (e.g., angiosperm SAM) there is a population of pluripotent cells that divide and maintain the meristematic stem cell niche (29). The Apical Cell has chiefly been defined by morphological characterization rather than direct experimental evidence. This is, to some degree, recursive: features at the centre of the notch are taken to be characteristic of the Apical Cell, and Apical Cell features are used to define the centre of the apical notch meristem (19, 48, 65).

The lateral merophytes are also called subapical cells, since they are morphologically similar to the Apical Cell and also divide in four planes (16, 17). The difference under the current model is that while subapical cells can divide to produce new subapical cells (i.e., lateral merophytes), they do not produce new Apical Cells. In dichotomous branching, a second Apical Cell is formed which then produces subapical cells between itself and its mother Apical Cell, resulting in apical notch bifurcation (47, 48). Experiments on misexpression and knockout lines for the stem-cell promoting peptide factor MpCLE2/CLV3 developed extra or reduced Apical and subapical cells respectively compared to wild-type plants (47), and knockout mutant lines of MpLOS1 had multiple Apical Cells (52) although no clear criteria was given as to how these cells were classified.

I tested the standard model of the apical notch experimentally, by ablating certain cells in the first row of apical notch cells (i.e., the Apical Cell and subapical cells) previously demonstrated to be crucial for meristem activity. This showed that the presence of the Apical Cell alone is not sufficient for apical dominance and that the total number of intact cells is not the only factor. What is important is that there are sufficient cells intact and in contiguous contact within the first row of the apical notch. The apex of the apical notch (i.e., the part containing the Apical Cell) seemingly has a greater functional capacity than the cells on the sides of the notch, since this central subpopulation alone could sustain meristem activity if no other apical notches were present in the gemma. This confirms that there is long-range communication between apical notches in a gemma.

When the first row of apical notch cells was ablated to remove one side and part of the notch centre, including the Apical Cell at the apex, there was still a sufficiently large contiguous subpopulation of apical cells to sustain meristem activity and apical dominance (Fig. 2*D, E*). After 4 days the apical notch had reorientated itself, with a new apex where the centre of the intact contiguous subpopulation of cells was originally. This was not emergence of a new meristem; instead it appears to be a case of meristem remodelling, where divisions of the subapical cells create a new apex of this apical notch, with an Apical Cell at its centre. These observations are

outside of the current model: a new Apical Cell is produced, but this is different from normal *de novo* meristem regeneration since it occurs even if another apical notch is present in the gemma.

These experiments demonstrate that the presence of a single Apical Cell is not the key factor for meristem activity and apical dominance. Rather than there being one essential Apical Cell surrounded by replaceable subapical cells, these results point towards there being a population of apical cells in the notch. What is important for meristem activity is that there is a sufficient number or "quorum" of these apical cells intact and in contiguous contact. The exact requirements for this quorum remain to be determined. Furthermore, the characteristic notch morphology emerges from self-organization of this quorum due to the biophysics of differential rates of cell division and expansion from the quorum centre to the edges (66). Under this updated model, the "Apical Cell" is not a fixed cell identity but instead emerges from the communication dynamics of the apical cell population within the notch. The "Apical Cell" identity is defined and regulated by the "subapical cells", as the latter are division products of the former. It is this feedback process (47, 48, 51) within the entire apical cell population that sustains meristematic activity and apical dominance.

The requirement for apical cells to be in contiguous contact points towards intra-notch communication occurring directly between the apical cells. Such paracrine signalling would provide a mechanism to regulate cell division rates from the apex to the sides of the notch, with a dilution effect informing cells on their relative position within the apical cell population. Paracrine communication within the apical cell population offers a basis for dichotomous branching, as previously suggested by Hirakawa and colleagues (47, 48). The two duplicated apical notch meristems can co-exist in close proximity without apical dominance because each consists of an equally sized quorum of apical cells, and this can be regulated since they each arise out of one contiguous population of apical cells. Post-branching regulation would then lead to these meristems becoming dormant (19) rather than losing meristem identity entirely. A quorum of pre-meristematic dividing cells may also be required for the production of meristematic apical cells, for example in the sporeling protonema to prothallus transition (10, 18) or during gemma formation. Understanding the processes underlying apical cell communication, quorum size and regulation will be relevant to branching and meristem specification in plant developmental biology more widely.

Partial Apical Notch Ablation Reveals that Meristem Regeneration is a Flexible Process

It is known that multiple new meristems can emerge from one gemma fragment or germinating spore, but that not all regions that begin the regeneration process form complete meristems (10), and not all complete meristems are divisionally active (19, 22, 23). This is attributed to some regenerating meristems having apical dominance or lateral inhibition effects on other emerging meristems (12). The basis for this dominance- size, relative positioning, timing- is unknown. One partial notch ablation experiment (Fig. 3, SI Appendix Fig. S5) revealed the importance of developmental stage and suggests that there are certain developmental thresholds that govern meristem dominance during regeneration. It also supports the conclusion that apical notch reorientation (Fig. 2*D, E*) was not due to regeneration of new meristem, since no new patches of cell division emerged elsewhere in the gemma fragment in those instances.

These experimental observations are noteworthy as they demonstrate that loss meristematic activity can be reversed, and that *de novo* meristem regeneration can be stopped even at a late stage. That the partially ablated notch could reassert apical dominance over larger patches of cell division suggests that developmental stage is more important than size. There may be certain threshold stages within the cellular reprogramming pathway for meristem regeneration, and since the partially ablated notch was already composed of apical cells it progressed faster through these stages. Regenerating regions that have passed specific thresholds could dominate other, less mature regions, while multiple regions could co-exist as long as they develop simultaneously (9, 10, 47, 48). This could provide a checkpoint mechanism for meristem regeneration and prevent too many regions emerging in too close proximity, and explain how bifurcating meristems can co-exist in close proximity, since they are of equal maturity.

Apical Dominance Signal Transport Across the Gemma

The partial notch ablation experiment shows that apical dominance in Marchantia is an active process that must maintained and can be temporarily disrupted or re-established. The apical notch meristem must emit a signal continuously to the rest of the plant to inhibit cell division and generate the thalloid growth pattern, since even a relatively brief interruption (<24 hours) see the initiation of widespread cell division.

Lasering incisions through the tissues around the apical notch demonstrates that apical cells produce a signal that flows outward to influence the rest of the gemma. Incisions parallel to the notch (SI Appendix Fig. S6*F-O*) show more cell division between the apical cells and the incision and more cell expansion distal to the incision. This was only observed when the apical cells closest to the incision were intact; when they were ablated there were no differences observed on either side of the incision. This was irrespective of the integrity of the cells at the apex of the notch, reinforcing that apical cells exist as a population rather meristem activity depending on a central Apical Cell.

The cells surrounding the apical notch are the youngest in the gemma and will be acquiring new fates (16, 17, 67), so it is expected that they would be integrating positional and developmental cues from the surrounding tissues. Changes in cell division and expansion notwithstanding, cells distal to the parallel incision did not enter the early, widespread cell division phase of meristem regeneration. This was the case whether the other apical notch meristem in the gemma was intact or excised. Therefore, while some signals flow directly from the notch, the apical dominance signal that represses new meristem emergence can be transported around the parallel incisions.

These findings led to further experiments to test which regions around the apical notch are important for conducting the apical dominance signal. This demonstrated that there is a spatial heterogeneity for transport of the repressive signal between connections formed from central and peripheral tissue. This was not dependent on how wide the connection is, as central connections 1-2 cells wide did not see new meristems emerge, whereas wider peripheral connections saw formation of new meristems elsewhere. This means that the apical dominance signal must be carried by a specific mechanism, and not all cells possess this mechanism.

Auxin is the prime candidate for this apical dominance signal. Auxin regulates processes such as apical dominance (7), meristem regulation (5, 6) and meristem regeneration (8) in all plants, including Marchantia (24, 25). Auxin is produced in the Marchantia apical notch and exported to the rest of the thallus. Surgically removing the apical notch removes this auxin source, causing auxin levels in the remaining thallus fragment to drop initially before rising again as new meristems regenerate (9). A reporter line marking expression of auxin biosynthesis gene MpYUC2 (68) shows that in 0dpg plants, MpYUC2 is expressed at the apical notch and throughout the gemma, but this becomes more focused around the apical cells in older gemmae (10). A reporter line marking expression of the canonical polar auxin transporter MpPIN1 (36, 46) shows that in 0dpg plants expression is focused around the apical notch, as well as around the gemma edge and in occasional cells across the centre of the gemma. In older gemmae, MpPIN1 expression becomes more focused around the apical cells (10). Collectively, these results show that the apical notch meristem acts as an auxin source in intact gemmae, but that auxin signalling is more widespread.

Canonical PIN distribution does not explain why the repressive signal is carried through central connections but not peripheral connections. MpPIN1 expression is high at the gemma edge, present in peripheral connections and absent from central connection cells (Fig. 4*C, F*). It may be the case that other auxin transporters are involved in transmission of apical dominance signal, for example short PIN, ABCB or AUX/LAX transporters (34, 36, 69). The involvement of other auxin transport mechanisms is supported by the finding that meristem regeneration still occurs in *Mppin1* mutants, albeit more slowly (56). The Marchantia non-canonical short PINs are promising candidates as recent work found that they are localized to the plasma membrane and are capable of auxin export. MpPINW has an asymmetric distribution in the plasma membrane, and this asymmetry appears to point centrally rather than peripherally in cells around the apical

notch expressing MpPINW (70). It should also be noted that the regions of localized cell division are marked by denser patches of MpPIN1 expression, suggesting that meristem regeneration involves reorganization of the auxin transport machinery (dashed circles, Fig. 4*F, I*) (56).

Balance between auxin sources and sinks is required for Marchantia meristem maintenance and regeneration

Auxin has a self-inhibitory effect on its own biosynthesis (35, 63) and elevated auxin levels prevent meristem regeneration (9, 21, 21). Therefore there needs to be balanced transfer of auxin between auxin sources, where the hormone is produced, and auxin sinks, where it can be degraded (22, 68, 71, 72). If the sink is too large compared to the source then apical dominance is lifted and new meristems emerge; if the sink is too small compared to the source then auxin build-up interferes with meristem activity. In Marchantia, an example of the former is seen in the short-term response to partial apical notch ablation (Fig. 3). Examples of the latter are apical notches isolated by peripheral connections (Fig. 4*D-I*), or by complete separation from the rest of the gemma fragment (SI Appendix Fig. S7). In these cases, the only available sink tissues are the cells immediately surrounding the apical notch. When this region is large and contains sufficient non-meristematic auxin sink tissue, then the isolated notch continues growing as normal (e.g., Fig. 4*E, F*). When there is a small region and there is insufficient sink tissue, then auxin accumulates, has a self-inhibitory effect and the apical notch loses meristem identity (Fig. 4 *H, I*, SI Appendix Fig. S7).

The auxin source-sink balance is also disturbed by removing both apical notches from a gemma. With the original auxin sources gone, auxin levels fall across the remaining fragment. Subsequently new sources (regions of cell division) emerge, until the balance is restored by the regeneration of full meristematic apical notches (9). A hypothesis that emerges from this is that, in a gemma fragment with both apical notches excised, if there are a greater pool of cells to act as auxin sources or sinks, then regeneration will proceed faster compared to fragments with fewer cells. The results of the experiments presented in Fig. 6 (see also SI Appendix Fig. S8) support this. An extreme version of the relationship between fragment size and meristem regeneration speed is the case of regeneration from a very small number of isolated thallus cells (10, 11, 73). In contrast to meristem regeneration from full gemmae, which is complete after ~5 days, isolated single cells or small clusters can take at least 5-7 days just for cell divisions to occur, another 4+ days for localized patches of cell division to stabilize, with recognizable notches only appearing after 14+ days. Meristem regeneration in the Marchantia gemma therefore presents a simplified system to study auxin self-inhibition and the balance between pools of cells acting as auxin sources and sinks. This system can be used as a basis for research into complex scenarios in plants, for example lateral organ formation (3, 7) or wounding responses (60, 61).

Conclusions

An outline model for the Marchantia apical notch meristem is given in Fig. 6. A population of apical cells are found in the first row of the notch, behind which are 2 rows of cells primed to acquire apical cell identity. The centre of the apical cell population forms the apex of the notch by having lower rates of cell division compared to the edges of the apical cell population. The apical cells produce intra-notch signals maintaining the apical cell quorum, short-range signals regulating cell division and expansion, and long-range signals sustaining apical dominance across the gemma. The apical dominance signal, likely auxin, is carried through the centre of the gemma and not the peripheral regions. If neither meristematic apical notch has a sufficient quorum of apical cells in contiguous contact, or if the apical dominance signal cannot flow to the rest of the gemma, then meristems emerge elsewhere in the gemma.

Materials and Methods

Vector constructs were built according to the Loop Assembly protocol (40, 74) from L0 parts, L1 and L2 plasmids in the OpenPlant Loop Assembly toolkit (40). The lines used for each figure are given in SI Appendix Supporting Text and full plasmid sequences for vector constructs

are given in SI Appendix Dataset S1. Plasmid vectors were transformed into Marchantia sporelings (Cam accession) by agrobacterium-mediated transformation according to the methods described previously (40, 41). T0 sporelings and subsequent G1 plants were grown at 21°C under continuous light (intensity= 150 µmol/m^2/s) on 1.2% w/v agar (Melford capsules A20021) plates of Gamborg B5 media with vitamins (Duchefa Biochemie G0210) prepared at half the manufacturer's recommended concentration and adjusted to pH 5.8. The media was supplemented with 100µg/ml Cefotaxime (BIC0111; Apollo Scientific, Bredbury, UK) to reduce bacterial or fungal contamination, and 20µg/ml Hygromycin (10687010; Invitrogen) for selection.

Imaging was carried out using a Leica Sp8 Confocal Microscope and LasX v3.5.7.23225, equipped with a hybrid detector and a pulsed white-light laser, with a HC PL APO 10x/0.40 CS2, a HC PL APO 20x/0.75 CS2 dry objective or a HC PL APO CS2 40x/1.30 oil objective. Excitation and collection settings for each fluorophore are given in SI Appendix Supplementary Information Text. Images were taken in photon counting mode with sequential acquisition, using bidirectional scanning at 600Hz and 2x line averaging. Time gating was active to supress autofluorescence. Maximum-intensity projections of the images were obtained from z-stack series, capturing z-slices at intervals between 1µm-12.5µm. The images were processed using the Leica LasX and Fiji/ImageJ software packages. Figures were produced using the QuickFigures (75) and Stitching (76) Fiji plugins.

Laser ablation experiments were performed using a Leica LMD6000 laser dissection microscope system equipped with a solid state 355nm cutting laser and controlled by Leica LMD6 software. Ablation performed under the 10x/0.3 HCX PL FL objective lens used settings 60 power, 45 aperture, 25 speed; ablation performed under the 40x/0.6 HCX PL FL objective lens used 60 power, 35 aperture, 20 speed. All gemmae used were taken directly from the gemma cup of the parent thallus (i.e., 0dpg). Intact gemmae were also taken from the same cups as controls to verify regular growth and marker expression. Gemmae were planted on 50mm agar plates and ablation and imaging performed directly on the plates to minimize mechanical disruption to the plants. For entire notch excision a 90µm diameter circle was drawn with its centre point at the notch apex, and all tissue within this circle was destroyed by laser ablation. All other cells or regions ablated are shown in schematics accompanying the relevant figures. Consistent gemma growth and marker signal responses were observed in at least three gemma replicates taken from two different lines, except in the case of Fig. 3/SI Appendix Fig. S5, where the ablation conditions for re-establishment of apical dominance by a partially ablated apical notch was observed once.

Propidium iodide (PI) staining was carried out to verify that the laser ablation settings did not cause widespread stress or uncontrolled damage to the gemma. WT and enhancer trap line gemmae were incubated in 10µl of 100µg/ml PI (Sigma) on a glass coverslip for 10 minutes at room temperature, ensuring that the gemma was not mechanically damaged or desiccated during incubation. Gemma staining and imaging was carried out before and/or after ablation in various combinations to ensure that any PI signal relating to cellular damage was solely due to laser ablation. All experiments showed that the laser settings used killed the targeted cells, that cellular damage was localized to the visible ablation zone and that ablation did not cause more widespread cell death or stress to the gemma (SI Appendix Fig. S9). Thus laser ablation microscopy is a suitable approach to study the microarchitecture of the apical notch region.

Acknowledgments

I would like to thank lab members Susanna Sauret-Güeto, Jenna Rever, Harriet Kempson, Sze Wai Tse and Eftychios Frangedakis for their advice and support. I especially thank Marius Rebmann for providing plant lines and valuable intellectual input. Mihails Delmans developed concepts related to these experiments. Jim Haseloff provided funding and influenced the development of this project. I thank Alexander Jones (Sainsbury Laboratory, Cambridge, UK) and Verena Kriechbaumer (Oxford Brookes University, Oxford, UK) for constructive comments on the manuscript, Satoshi Naramoto (Hokkaido University, Japan) for providing the 5' promoter sequence for MpYUC2 to create the vector construct, and Kuan-Ju Lu (National Chung Hsing

University, Taiwan) for providing data on Marchantia short PIN expression. This work was funded as part of the Biotechnology and Biological Sciences Research Council/ Engineering and Physical Sciences Research Council OpenPlant Synthetic Biology Research Centre Grant BB/L014130/1 to Prof. Jim Haseloff and Biotechnology and Biological Sciences Research Council grant BB/F011458/1 for confocal microscopy to Prof. Jim Haseloff.

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Figures and Tables

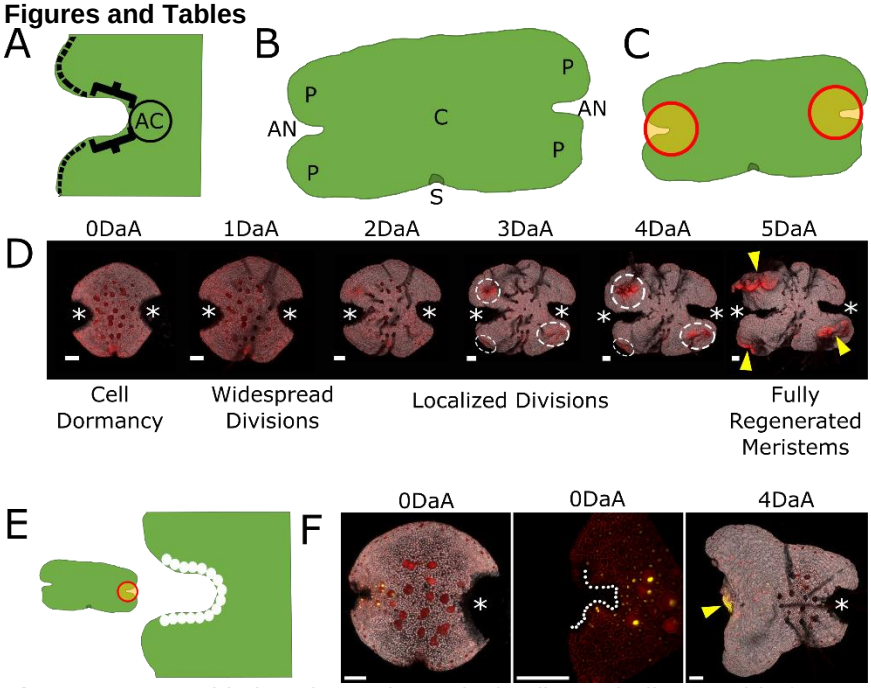


Figure 1. Laser ablation shows that apical cells are indispensable for meristem function. (A) Apical notch schematic. AC marks the Apical Cell at the apex (centre) of the notch, flanked by subapical cells (brackets) extending along the sides of the notch. These cells comprise the first row of cells. Further out of the notch the first row of cells are comprised of non-meristematic margin tissue (dashed line) (10). (B) Gemma schematic showing the two apical notches (AN) at either end. To the sides of the notches are the peripheral regions (P) and behind the notches is the central part of the gemma (C). The stalk scar (S) is where the gemma was attached to the parent thallus within the gemma cup. (C) Schematic of the ablation pattern (orange bounded by red) for complete excision of all notches. (D) Time course of a gemma fragment expressing an mScarlet cell membrane marker following complete notch excision. The four main phases of meristem regeneration are described. Excised notches are marked by asterisks. Cell division can be seen by increased density of red marker signal, localized patches of cell division are marked by dashed circles and new, fully regenerated meristems are marked with yellow arrows. Images were acquired at the indicated time-points after meristem removal (days after ablation, DaA). (E) shows a schematic of the ablation patterns used in the fine scale gemma ablation experiment. Orange bounded by red denotes excised notch, white circles indicate ablated cells. (F) shows, from left to right, the 0DaA whole gemma (asterisk marking excised notch), a close up of the 0DaA notch (ablated cells marked by white circles) and the 4DaA gemma (yellow arrow marks regenerated meristem). This demonstrates that ablating the first row of cells in the apical cells is sufficient to induce meristem regeneration, which occurs just behind the ablated notch. Gemma shown is from the enhancer trap notch/meristem marker line ET239-P153. See SI Appendix Fig. S2*B* for the full time series. Confocal images show membrane marker expression in red, nuclear mVenus enhancer trap signal in yellow and chlorophyll autofluorescence in grey. Scale bars= 100µm.

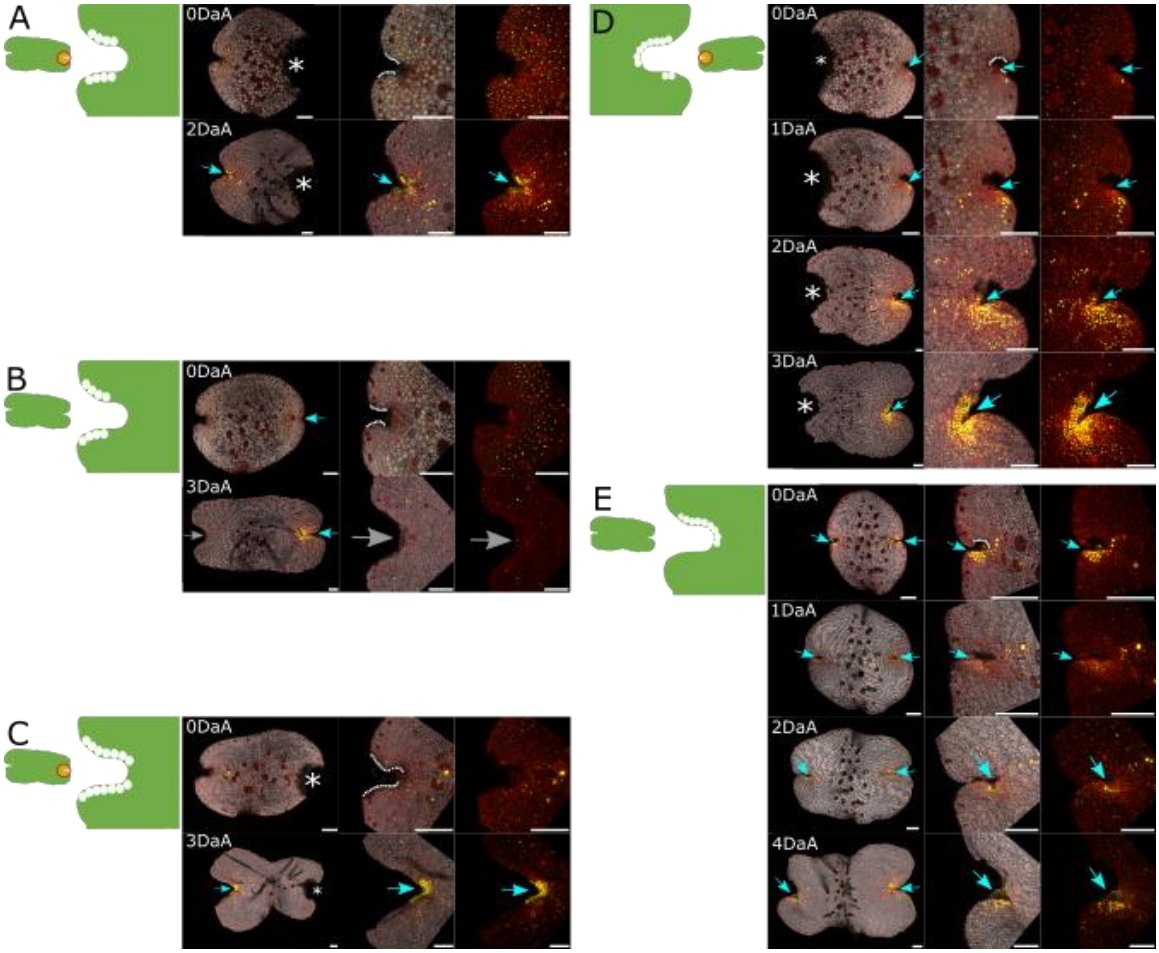


Figure 2. The notch apex, containing the Apical Cell, can sustain meristem activity only if the other apical notch is excised, but notch reorientation can occur after partial ablation of the notch apex, independent of the status of the other apical notch in the gemma. Ablation of the notch sides, leaving the notch apex (including the Apical Cell) intact, did not disrupt meristem activity if the other apical notch in the gemma was excised (A), but when the other apical notch was intact then meristem activity ceased (B). (C) Meristem activity continued even if few cells were left intact at the apex, if the other notch was excised. (D) and (E) show that partial notch ablation, such that the intact subpopulation of apical cells spans the apex and one side of the notch but excludes the Apical Cell location, results in reorientation of the apical notch. Meristematic activity continued, as indicated by mVenus expression. The notch changes shape, forming a new apex corresponding to the centre of the original intact subpopulation of apical cells. Reorientation occurs whether the other notch in the gemma was excised (D) or intact (E). In each sub-figure a schematic of the ablation pattern is shown with white circles marking the location of ablated cells and orange bounded by red denoting excised tissue. Time courses are given in the format whole gemma, notch close up, notch close-up without chlorophyll autofluorescence channel. Asterisks mark excised notches, blue arrows denote active meristematic apical notches, grey arrows mark apical notches that have lost meristematic activity. In the 0DaA images, white circles mark ablated cells. The enhancer trap notch/meristem marker lines used were ET239-P21 (A, B, D, E) and ET239-P153 (C). Scale bars=100µm.

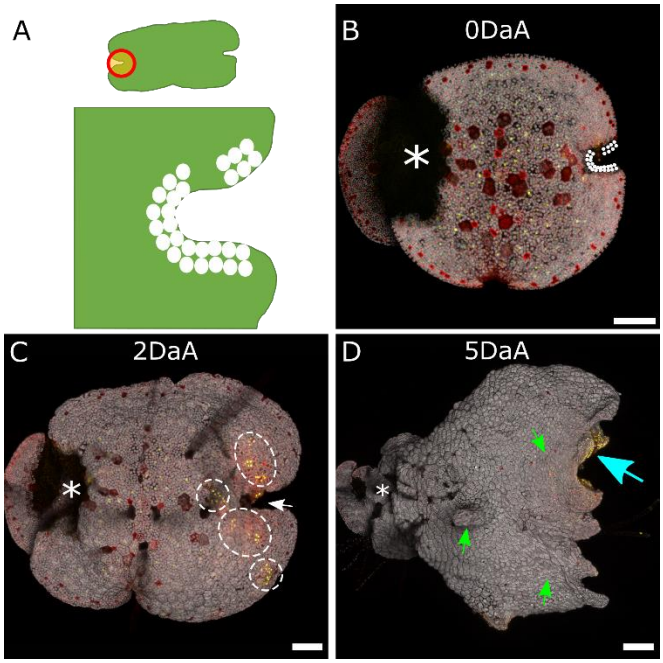


Figure 3. Time course demonstrating re-establishment of apical dominance by a partially ablated apical notch. (A) is a schematic of the ablation pattern used. White circles mark ablated cells, orange bounded by red marks excised tissue. (B) is the gemma immediately after laser ablation. White circles mark the position of ablated apical cells, asterisk indicates the entirely excised apical notch. (C) At 2DaA patches of localized cell division and marker signal appeared (dashed circles), even though the original, partially ablated notch still displayed marker signal (white arrow). (D) By 5DaA, the original partially ablated notch was meristematically active (blue arrow), as indicated by the presence of dense marker signal, and the apex of this notch had reorientated. The areas that were patches of cell division had no marker signal (i.e., no cell division activity) and remained as protrusions on the gemma surface (green arrows). The complete course is given in SI Appendix Fig. S5. The enhancer trap notch/meristem marker line used was ET239-P125. Scale bars=100µm.

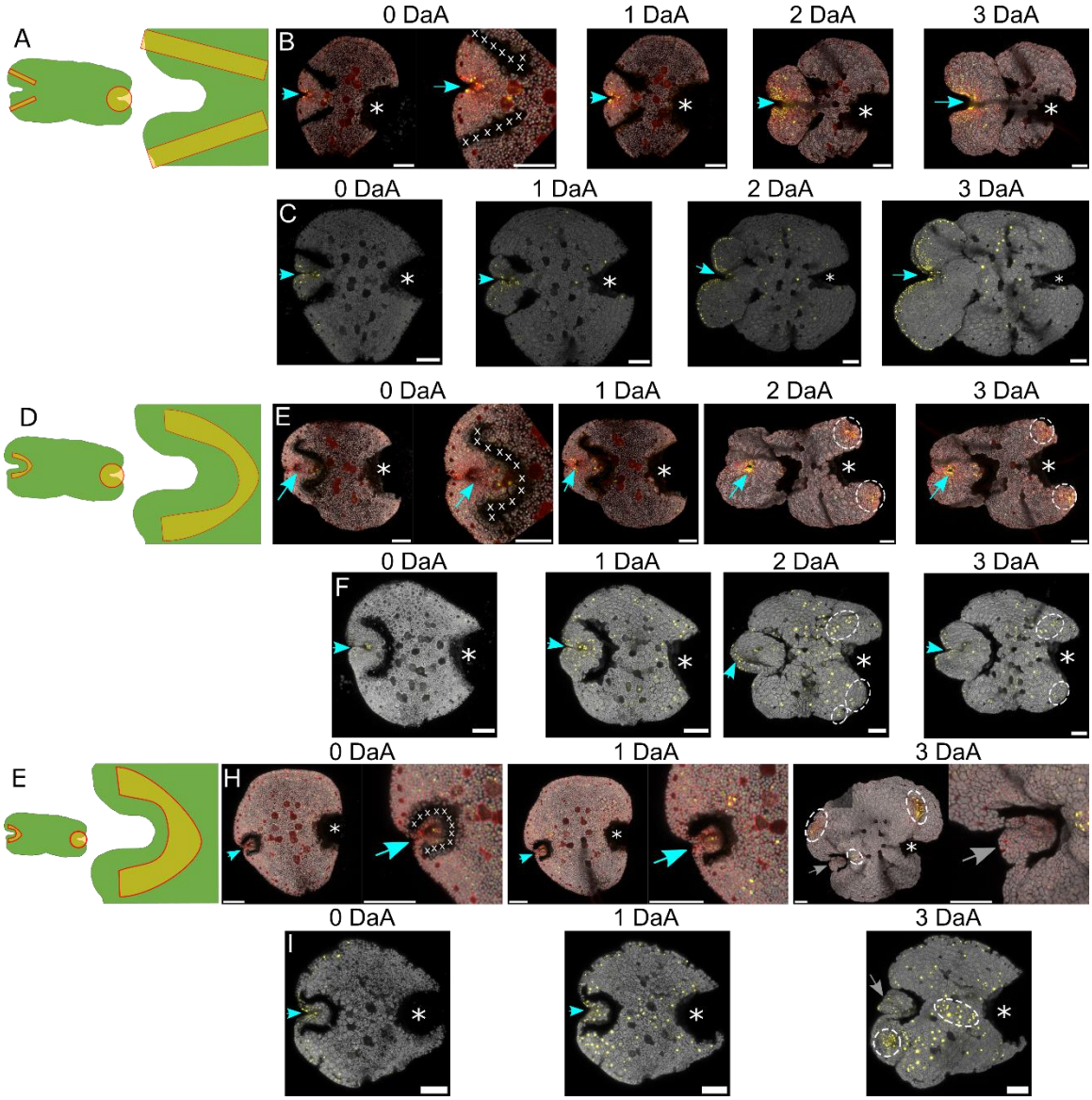


Figure 4. Ablating tissue around the apical notch demonstrates that there is heterogeneity across the gemma for apical dominance signal transport. (A) shows a schematic of the ablation pattern used in time courses (B) and (C). The intact apical notch maintained activity (blue arrows). No new meristems emerged in the gemma fragment attached to this notch by a central connection. (C) shows MpPIN1 expression at the notch and the gemma edge with little expression in the centre of the gemma. MpPIN1 marker signal was absent in the central connection. (D) shows a schematic of the ablation pattern used in time courses (E) and (F). The apical notch continued activity (blue arrows), however meristem regeneration (dashed circles) occurred in the gemma fragment attached to this notch by the peripheral connections. In (E) this was marked by the appearance of new enhancer trap mVenus marker signal, while in (F) this was indicated by proliferation of MpPIN1 expression throughout the gemma, and later by clusters of MpPIN1 expression. Though MpPIN1 signal was visible in the peripheral connection early on in time course (F), this connection apparently did not conduct apical dominance signal from the intact notch. This suggests that canonical PIN-type auxin transporters are not responsible for transporting apical dominance signal. (G) shows a schematic of the ablation pattern used in time courses (H) and (I), where the gemma was ablated to leave only a small amount of tissue

surrounding the intact apical notch. By 3DaA, new meristematic regions emerged (dashed circles). The intact apical notch was active initially, as indicated by enhancer trap marker expression (blue arrows in (H)) but by 3DaA this notch was inactive (grey arrows in (H)), with the cells losing marker signal, ceasing division and expanding. In the MpPIN1 marker line time course, loss of expression was observed at the inactive notch (grey arrows, (I)). This suggests that because peripheral connections cannot export the apical dominance signal, without a sufficient pool of cells around the notch to act as a sink the apical notch self-inhibits. Asterisk marks entirely excised notch, blue arrows denote active apical notches, grey arrows denote inactive apical notches, dashed circles mark patches of localized cell division. In outline schematics (A, D, G) X marks incision ablations, orange bounded in red denotes excised tissue. Gemmae shown are from the enhancer trap notch/meristem marker line ET239-P153 (B, E), ET-239-P125 (H) or MpPIN1 marker line with mVenus expression in yellow (C, F, I). Scale bars= 100µm.

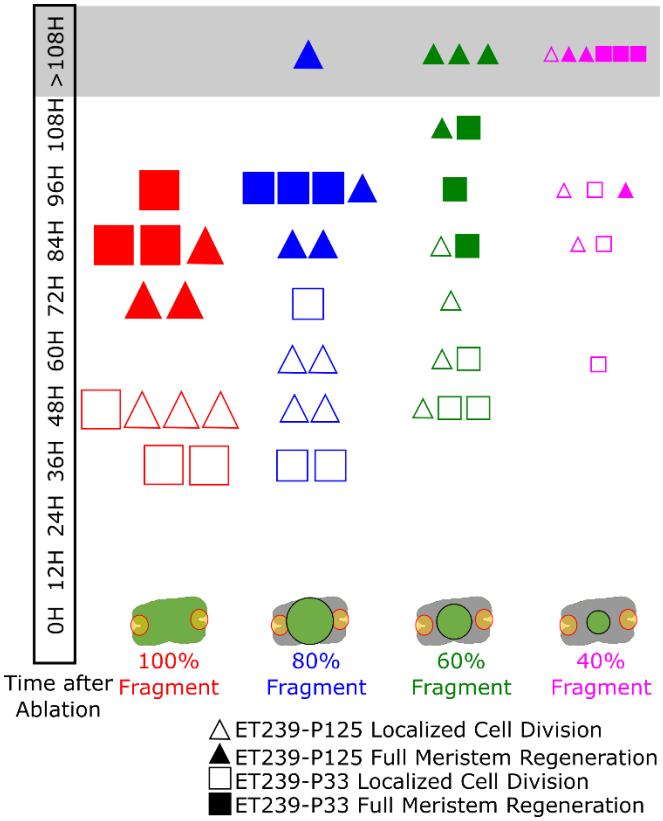


Figure 5. A 108-hour time course using two different enhancer trap apical notch/meristem marker lines shows that larger gemma fragments show faster emergence of patches of localized cell division and faster regeneration of full meristems. All 100% gemma fragments showed full meristem regeneration within the time course, as did all but one 80% fragment (shown by symbol in the grey >108H box). All 60% fragments had patches of localized cell division and marker signal emerge during the time course, but full meristem regeneration did not occur in all replicates. Only one of the 40% fragments regenerated full meristems while one did not even form localized patches of cell division with marker signal. A schematic of the ablation pattern used is given at the bottom, with orange bounded by red indicating an entirely excised apical notch. The black circle shows the laser ablation circle trace used, with the circle diameter calculated as a percentage of the original notch-notch distance. Open symbols denote appearance of patches of localized cell division with enhancer trap marker signal; closed symbols denote when full meristem regeneration occurred (defined as notch morphology within a dense patch of marker signal). An example time course is given in SI Appendix Fig. S8.

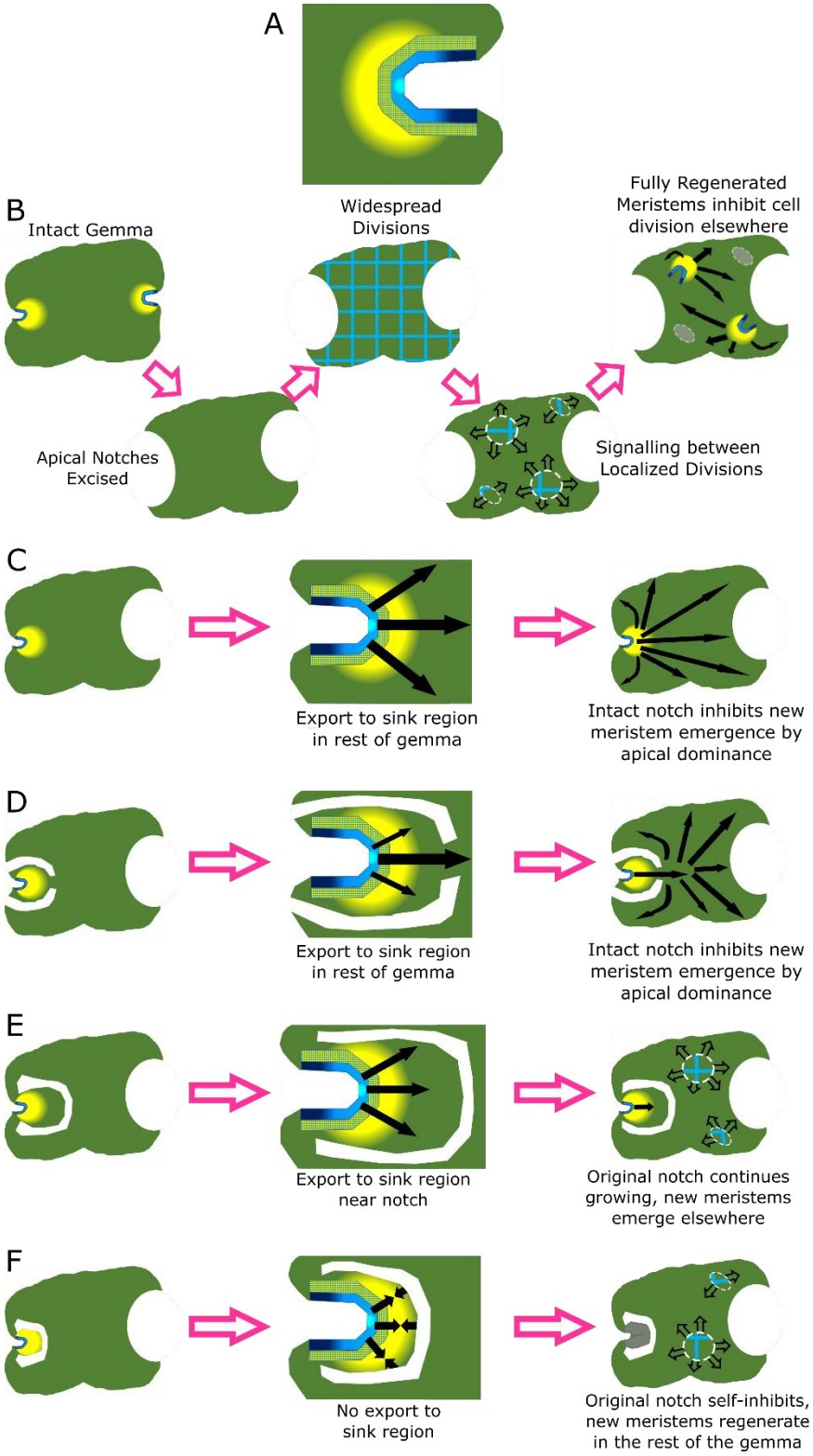
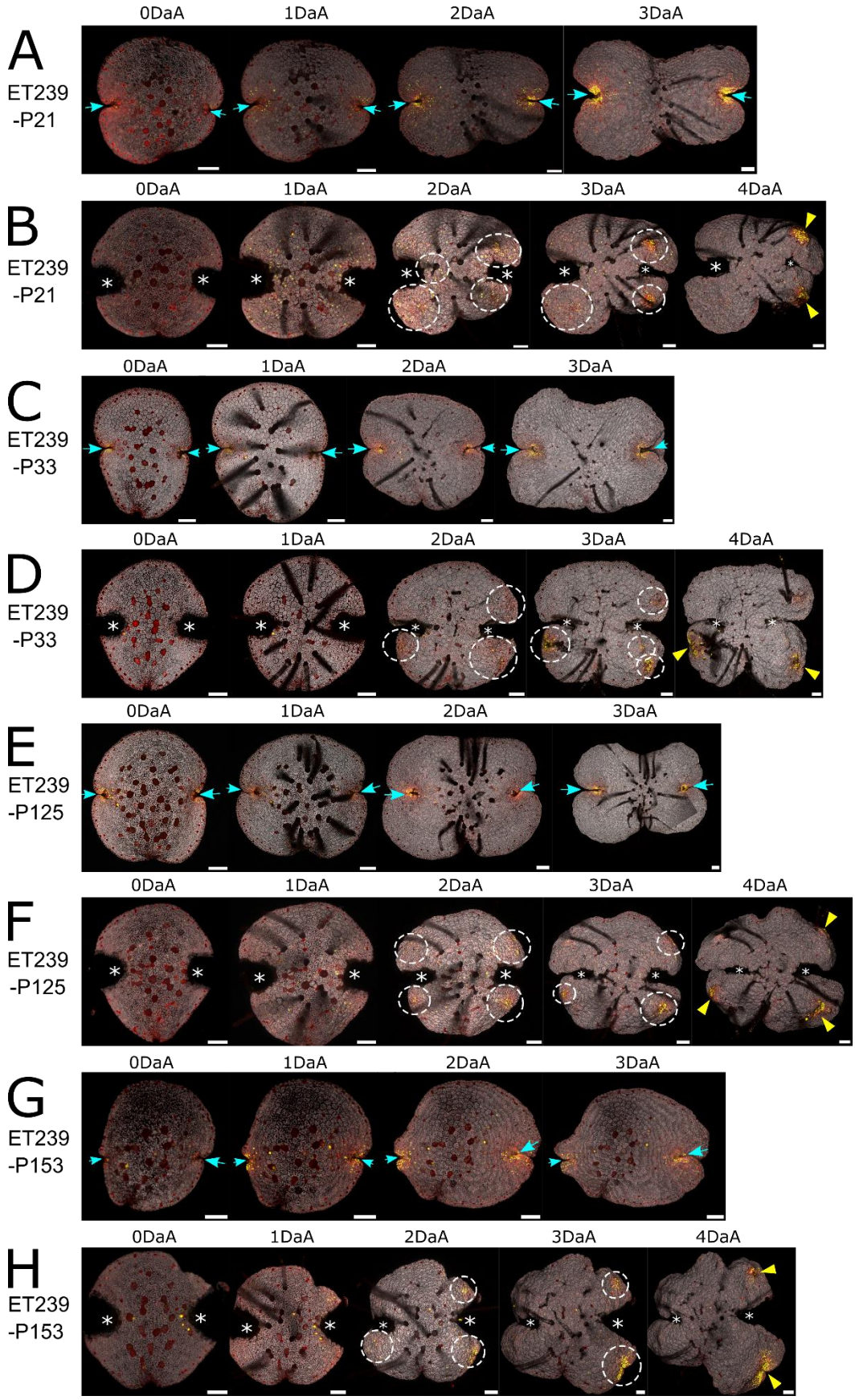


Figure 6. A model of meristem organization, regeneration and signalling in Marchantia. (A) The first row of cells (in blue) is the critical meristem organizer. The cells at the centre of this

population are distinct from the cells towards the edge (blue gradient). The cells in the two rows behind are primed to regenerate new meristem (blue hatching). The apical cells emit a signal regulating cell division and expansion in the surrounding region (yellow gradient). (B) Process of meristem regeneration after both apical notches are entirely excised from a gemma. Initially there are widespread cell divisions (blue grid), then cell divisions are restricted to localized patches (circles) which communicate (open arrows). Eventually one or more of these patches passes the threshold to full meristem status and produces a repressive auxin signal (closed black arrows) that maintains apical dominance and inhibits cell division elsewhere. (C) When one apical notch is intact, it emits an apical dominance auxin signal (closed black arrows) that prevents the emergence of any new meristems. (D) When one apical notch is intact and linked to the rest of the gemma fragment through a central connection, then apical dominance signal is exported (closed black arrows) and no new meristems emerge. (E)-(F) When one apical notch is intact and linked to the rest of the gemma fragment through a peripheral connection, no apical dominance signal is exported (black arrows) and new meristems emerge elsewhere in the gemma fragment (circles). If the intact apical notch is surrounded by sufficient cells to act as an auxin sink then it continues to be active (D). If the intact apical notch is not surrounded by sufficient sink tissue, then auxin builds up and the meristem becomes inactive (marked in grey) due to self-inhibition (E).


A
ET239
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0DaA
1DaA
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B
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4DaA
C
ET239
-P33
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1DaA
2DaA
3DaA
D
ET239
-P33
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2DaA
3DaA
4DaA
E
ET239
-P125
0DaA
1DaA
2DaA
3DaA
F
ET239
-P125
0DaA
1DaA
2DaA
3DaA
4DaA
G
ET239
-P153
0DaA
1DaA
2DaA
3DaA
H
ET239
-P153
0DaA
1DaA
2DaA
3DaA
4DaA

Fig. S1. Apical notches and meristem regeneration in the enhancer trap Apical Notch/Meristem marker lines used in this study. (A) is an intact gemma from line ET239-P21 imaged daily from 0dpg until 3dpg. (B) is a gemma from line ET239-P21 with both notches entirely excised by laser ablation, imaged daily from 0DaA until 4DaA. (C) is an intact gemma from line ET239-P33 imaged daily from 0dpg until 3dpg. (D) is a gemma from line ET239-P33 with both notches entirely excised by laser ablation, imaged daily from 0DaA until 4DaA. (E) is an intact gemma from line ET239-P125 imaged daily from 0dpg until 3dpg. (F) is a gemma from line ET239-P125 with both notches entirely excised by laser ablation, imaged daily from 0DaA until 4DaA. (G) is an intact gemma from line ET239-P153 imaged daily from 0dpg until 3dpg. (H) is a gemma from line ET239-P153 with both notches entirely excised by laser ablation, imaged daily from 0DaA until 4DaA. In all lines, mVenus (in yellow) is strongly expressed in the dense cluster of cells in and around the intact meristematic apical notch, marked by blue arrows. Reappearance of densely clustered mVenus expression following apical notch removal occurs in all lines (marked by dashed circles) and indicates patches of localized cell division, with new regenerated meristems marked by yellow arrows. All lines express a constitutive mScarlet cell membrane marker (in red) and chlorophyll autofluorescence is shown in grey. The ablation pattern used is as in Fig. 1C. Scale bars= 100µm.

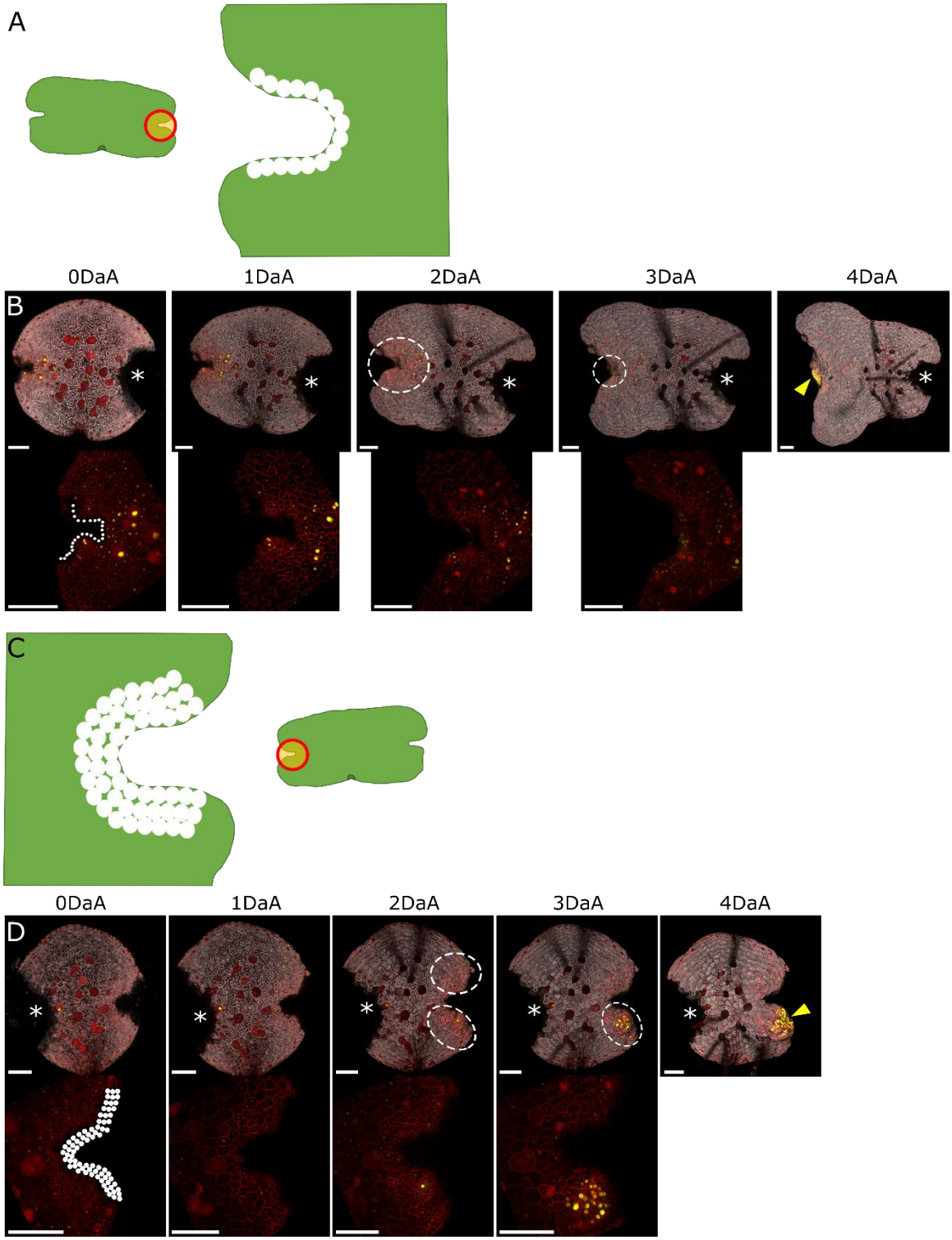


Fig. S2. Fine-scale ablation of the apical notch shows that the first three rows of cells are critical in determining meristem regeneration and its location. (A) and (C) are schematics for the ablation patterns used to ablate of the first row and first three rows of cells in the apical notch, respectively. White circles indicate ablated cells, orange bounded by red shows complete tissue

excision. (B) and (D) are gemma from the enhancer trap notch/meristem marker line ET239-P153 imaged daily during a time course from 0DaA until 3DaA (notch close-up) or 4DaA (whole gemma). White circles mark the position of cells removed by laser ablation in the notch close-up images of 0DaA gemma. One notch in the gemma was entirely excised (marked by asterisk). (B) shows a gemma where only the first row of cells in the notch was ablated. This is sufficient to induce meristem regeneration by 4 DaA, but this occurs at the ablated notch, as seen by mVenus signal marking the localized patch of cell division (dashed circle) and fully regenerated meristem (yellow arrow). (D) shows a gemma where the first three rows of cells in the notch were ablated. This was sufficient to allow patches of localized cell division (dashed circles) and eventually meristem regeneration (yellow arrow) to occur elsewhere in the gemma, away from the original notch. Scale bars= 100μm.

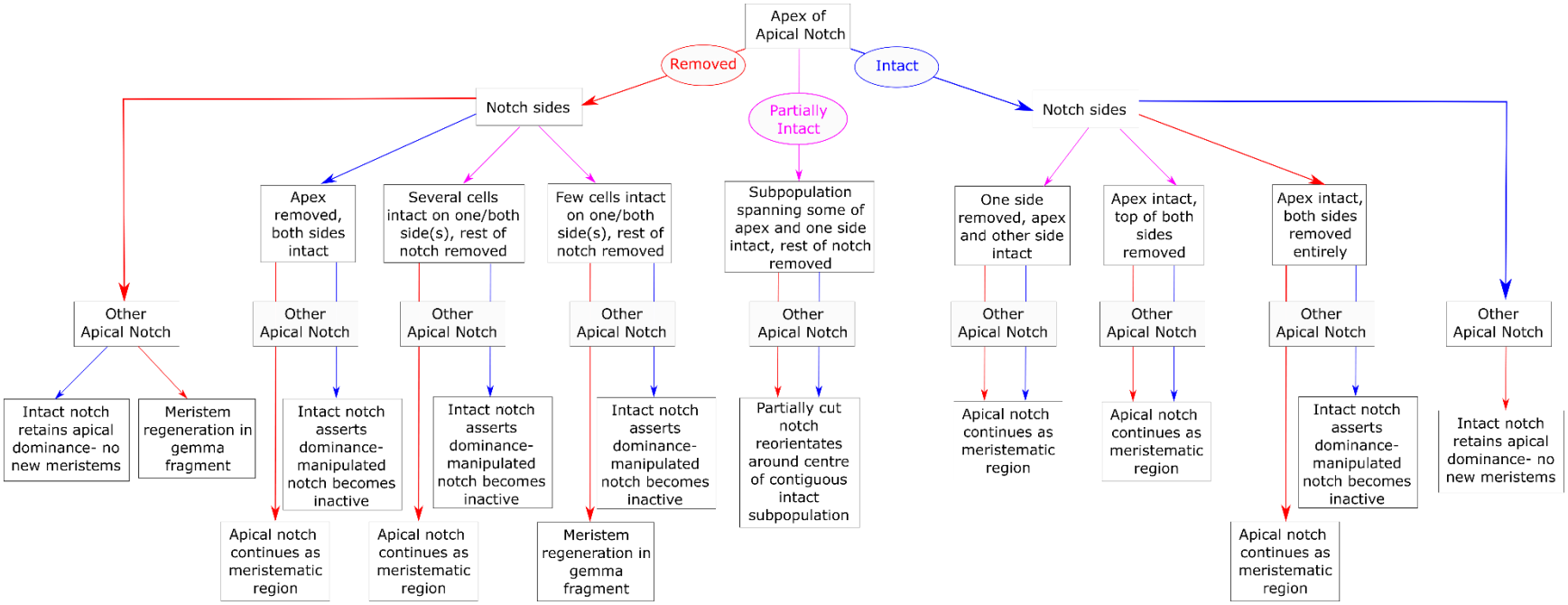
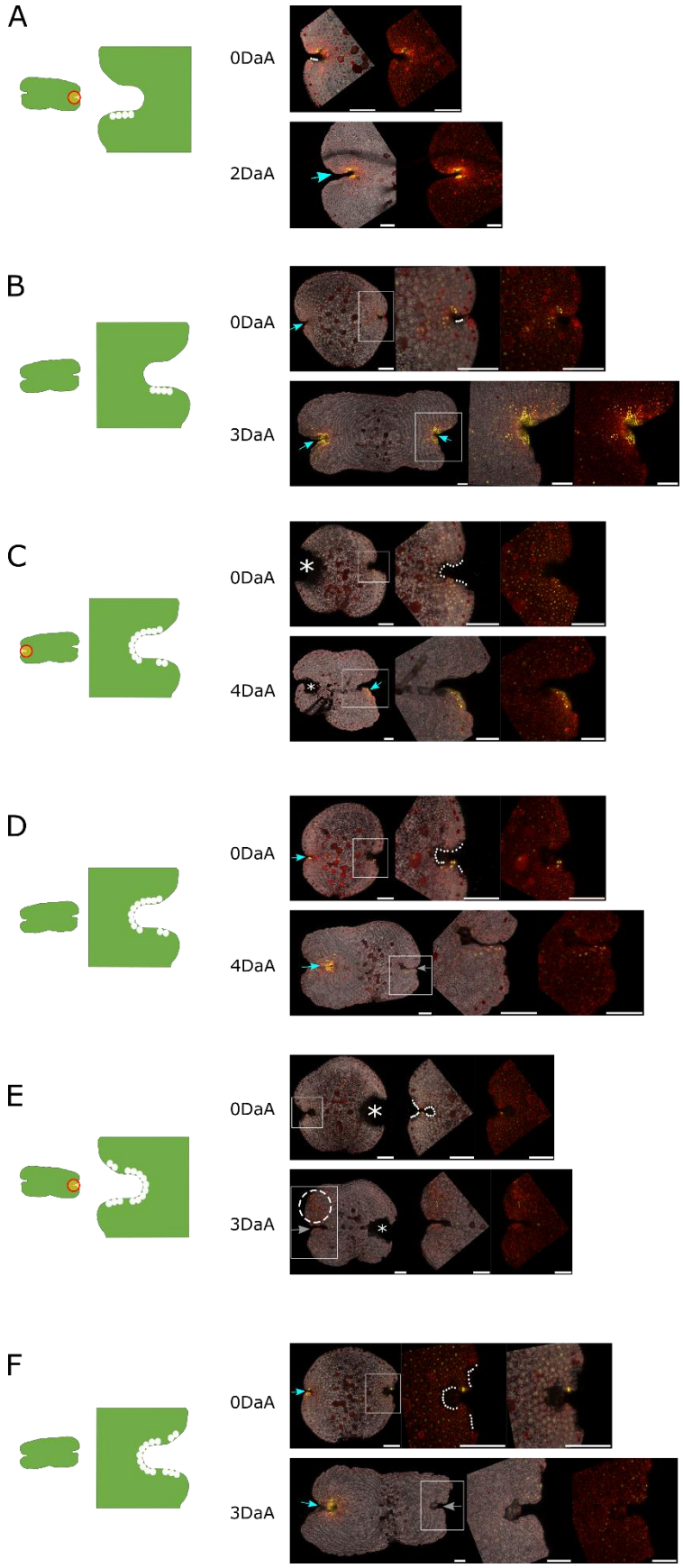

Apex of Apical Notch
Removed
Partially Intact
Intact
Notch sides
Apex removed, both sides intact
Several cells intact on one/both side(s), rest of notch removed
Few cells intact on one/both side(s), rest of notch removed
Subpopulation spanning some of apex and one side intact, rest of notch removed
Notch sides
One side removed, apex and other side intact
Apex intact, top of both sides removed
Apex intact, both sides removed entirely
Other Apical Notch
Intact notch retains apical dominance- no new meristems
Meristem regeneration in gemma fragment
Intact notch asserts dominance-manipulated notch becomes inactive
Apical notch continues as meristematic region
Partially cut notch reorientates around centre of contiguous intact subpopulation

Fig. S3. Flow chart of outcomes of ablation experiments. The flow chart progresses from the status of the apex of the apical notch, through the status of notch sides and to the status of the other apical notch meristem in the gemma. Red arrows indicate complete excision, magenta arrows indicate partial ablation, blue arrows indicate where a structure is left intact.


A
0DaA
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0DaA
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C
0DaA
4DaA
D
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E
0DaA
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F
0DaA
3DaA

Fig. S4. Precise partial ablations of the apical notch. In each sub-figure a schematic of the ablation pattern is given (whole gemma and notch close-up), with white circles marking the location of ablated cells and orange bounded by red denoting excised tissue. Images of the 0DaA gemma are shown on the top row of each sub-figure (whole gemma, close-up of the apical notch area marked in the white box, same close-up without the chlorophyll autofluorescence channel) and the bottom row shows the same gemma at the indicated time point in the same format. Asterisks mark entirely excised notches, blue arrows mark active apical notches, grey arrows mark apical notches that have lost meristematic activity and dashed circles mark patches of localized cell division indicative of meristem regeneration. (A) and (B) demonstrate that ablating one side of the apical notch does not disrupt meristematic function irrespective of whether the other notch in the gemma is intact or not. Ablation of the notch, including the apex, leaving only a portion of one side intact does not disrupt meristem activity if the other notch is excised (C) but if the other notch is intact then meristematic activity ceases (D). Leaving only a few cells intact on both sides of the notch does not sustain meristem activity, irrespective of whether the other notch is excised (E) or intact (F). If the other notch is excised then meristem regeneration occurs elsewhere in the gemma fragment (dashed circle, E). This is despite the total number of intact apical cells across the whole notch being greater than in the ablation experiment shown in (C). The enhancer trap lines used were ET239-P125 (A), ET239-P21 (B, C, E, F) and ET239-P153 (D). Scale bars= 100μm.

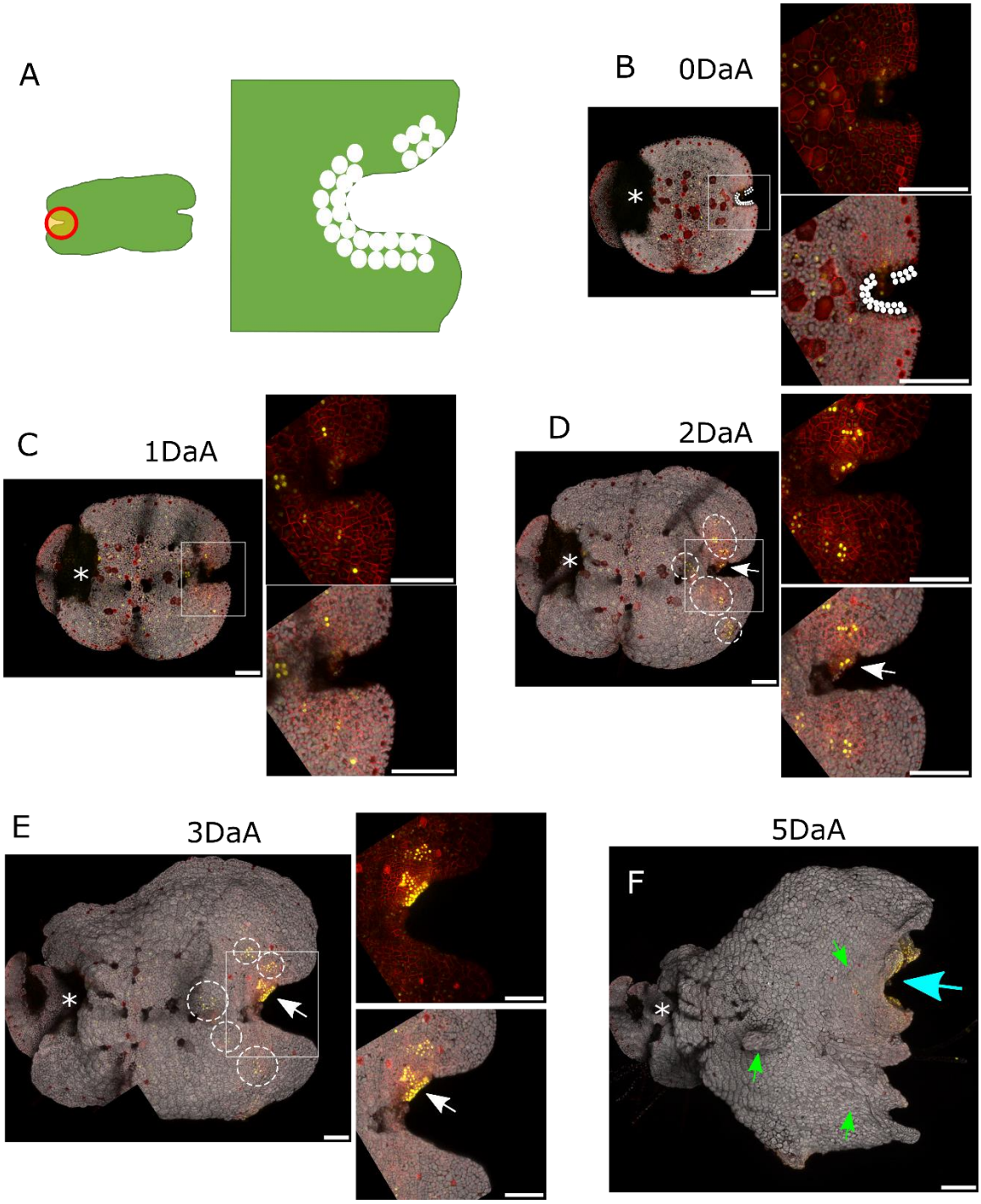


Fig. S5. Re-establishment of apical dominance by a partially ablated apical notch. (A) shows a schematic of the ablation pattern used, with white circles marking ablated cells, orange bounded by red denoting excised tissue. (B)-(F) shows a time course of a gemma from the enhancer trap notch/meristem marker line ET239-P125. The images shown are of the whole gemma, together with close-ups of the ablated apical notch region in the white square (shown with and without the chlorophyll autofluorescence channel). Asterisks mark the entirely excised apical notch. (B) is the 0DaA gemma, with white circles marking ablated apical cells. (C) is the

same gemma imaged at 1DaA, (D) at 2DaA, (E) at 3DaA. (F) is the whole gemma imaged at 5DaA. Although the original, partially ablated notch remained active (as indicated by the continuation of mVenus marker signal, white arrows), new patches of cell division emerged at 2DaA (D, dashed circles), with some persisting at 3DaA (E, dashed circles). However, by 5DaA (F) the original notch had reformed and re-established apical dominance (marked by blue arrow), as demonstrated by this notch being the only region of the gemma with mVenus marker signal. The areas that were patches of localized cell division stopped dividing (indicated by loss of mVenus signal) and remained as protrusions on the gemma surface (green arrows). These observations were recorded in one gemma replicate. Scale bars= 100µm.

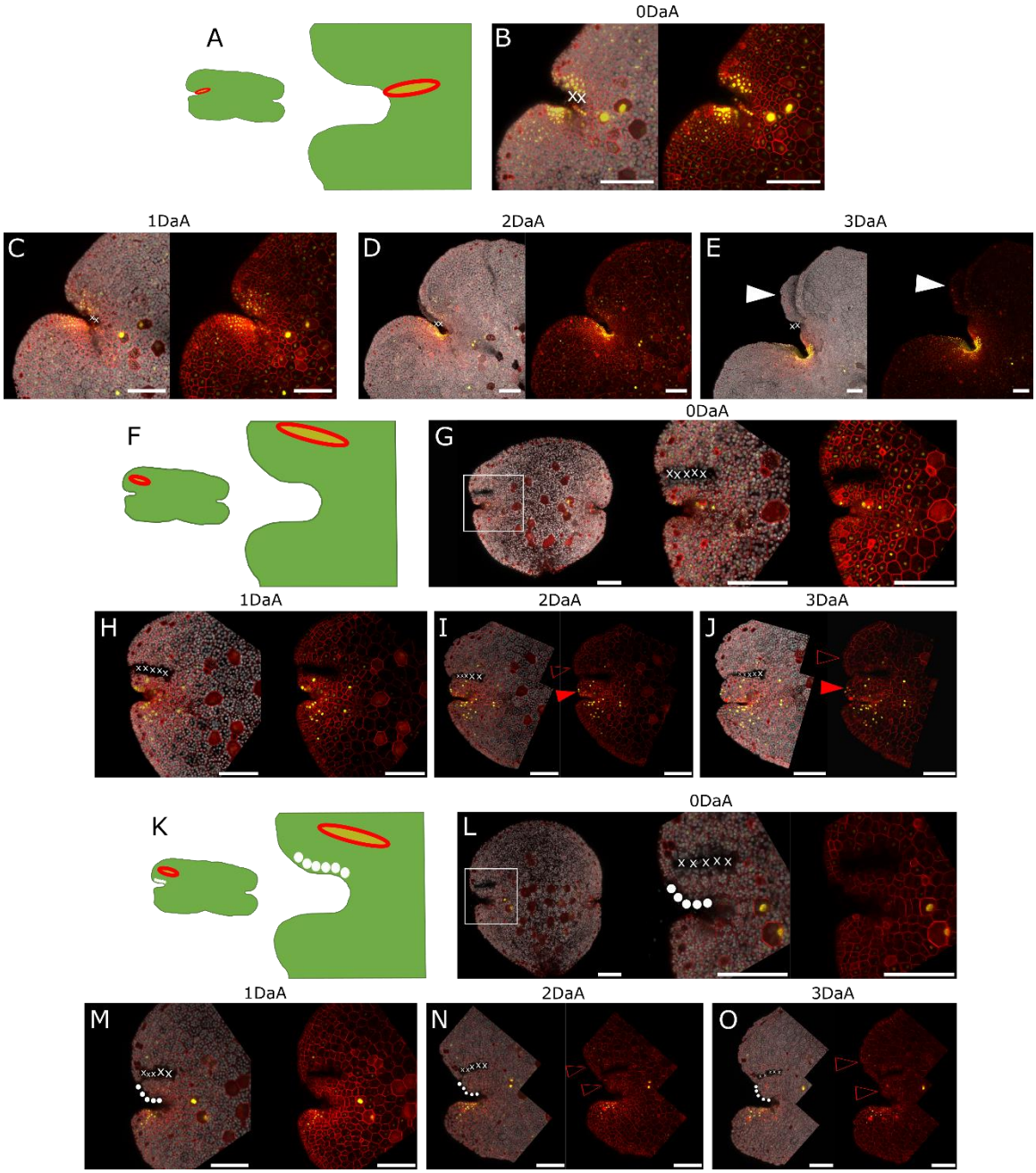
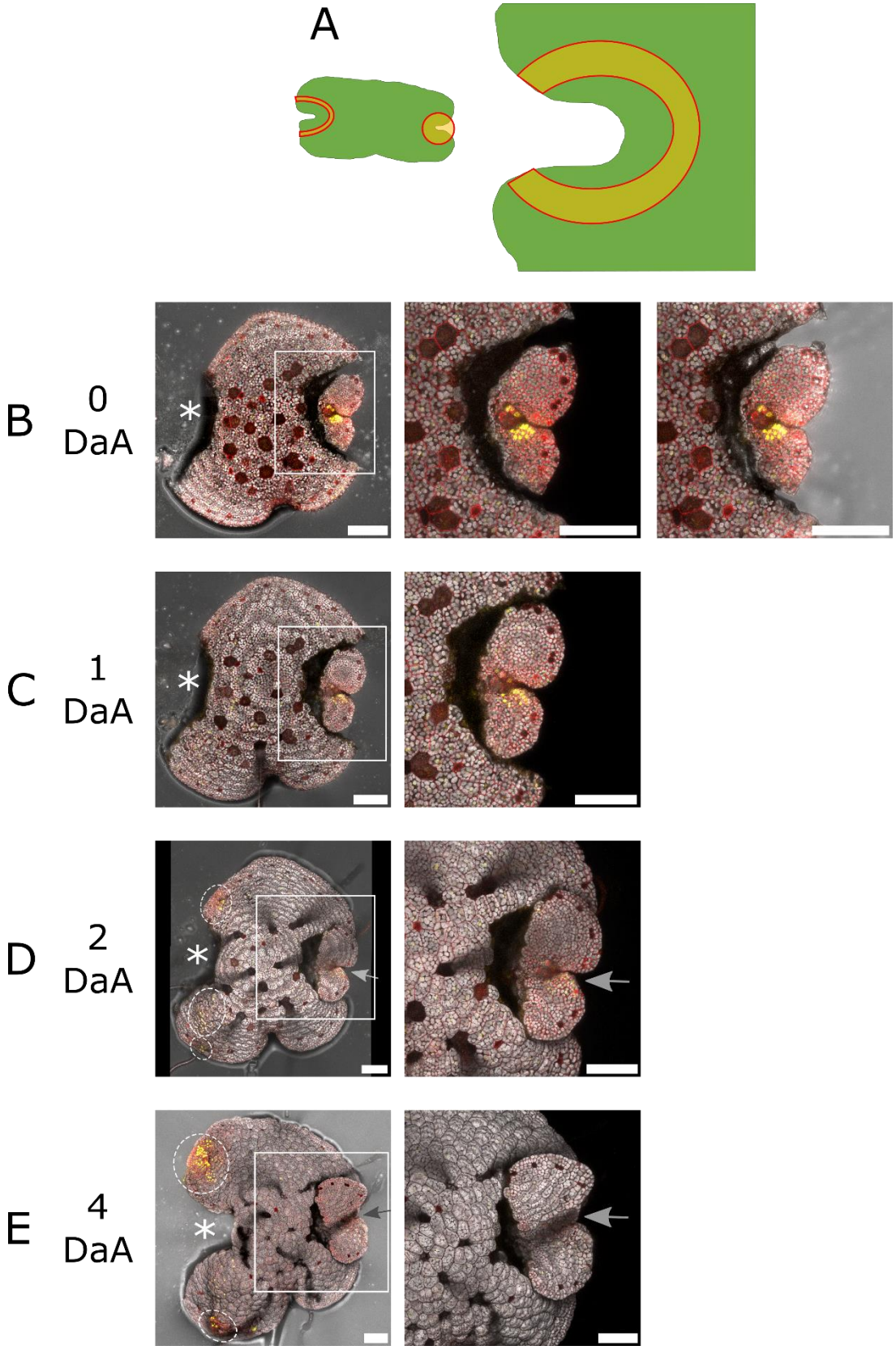


Fig. S6. Ablating an incision perpendicular to the apical notch has little effect, whereas ablating an incision parallel to the apical notch alters the balance between cell division and expansion either side of the incision site. (A) is a schematic of the perpendicular ablation incision pattern, with orange bounded by red denoting excised tissue. (B) shows a close-up of the ablated notch, with white X marking ablated tissue. (C) is the same gemma imaged at 3DaA, (D) at 2DaA, (E) at 3 DaA. Ablation perpendicular to the apical cells had no obvious effect on notch function, other than the z-axis thallus split (10) in the gemma appearing earlier on the ablated side of the notch (white triangle), at 3DaA. (F) and (K) are outlines of the parallel ablation incision patterns, with orange bounded by red denoting excised tissue and white circles marking ablated cells. (G)-(J) are time courses from 0DaA until 3DaA of a gemma ablated in the patterns shown in (F), with white X symbols marking ablated tissue in (G). Proximal to the incision there was

increased cell division (closed red triangle), whereas distal to the incision there was less cell division and more cell expansion (open red triangle), compared to the equivalent position on the non-ablated side of the notch. (L)- (O) is a daily time course from 0DaA until 3DaA of a gemma ablated as in (K), where in addition to the tissue ablated parallel to the apical notch (white X symbols in L) the apical cells proximal to the incision had also been ablated (white circles in L). In this case the cells on both sides of the incision showed reduced division and increased cell expansion (open red triangles) compared to the equivalent position on the non-ablated side of the apical notch. Gemma images are given in the format whole gemma (G and L only), notch close-up, notch close-up without the chlorophyll autofluorescence channel. Gemmae used were from the enhancer trap notch/meristem marker lines ET239-P125 for the perpendicular ablation experiment (B-E) and ET239-P153 for the parallel ablation experiments (G-J and L-O). Scale bars= 100µm.


A
B
0
DaA
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4
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Fig. S7. Entirely ablating the tissue surrounding the apical notch results in the isolated notch losing meristematic activity. (A) shows a schematic of the ablation pattern used, where one notch was entirely excised and laser ablation used to excise all tissue surrounding the other notch (excised tissue shown in orange bounded by red). The isolated apical notch itself was intact and surrounded by a small amount of tissue. The confocal mages show a gemma from the enhancer trap notch/meristem marker line ET239-P21 with one apical notch isolated using this ablation pattern, imaged daily during a time course from 0DaA (B) through 1DaA (C), 2DaA (D) until 4DaA (E). The whole gemma image includes transmitted light PMT channel (gain=315) in addition to the mVenus (yellow), mScarlet (red) and chlorophyll autofluorescence emission channels; the white box in the whole gemma image corresponds to the area shown in close-up. The 0DaA close-up includes the transmitted light channel image to confirm that the apical notch was fully isolated from the rest of the gemma fragment by destruction of the intermediate tissue. Appearance of dense regions of new mVenus signal (dashed circles) shows that this isolation removed the effects of apical dominance on the rest of the gemma and allowed patches of localized cell division to emerge. The isolated notch itself showed loss of mVenus signal, cell division ceased and the cells expanded, indicating that this region lost meristematic activity (grey arrows). This suggests that without a sufficient pool of cells in the isolated portion to act as an auxin sink, build-up of auxin causes the isolated notch to self-inhibit. Scale bars= 100µm.

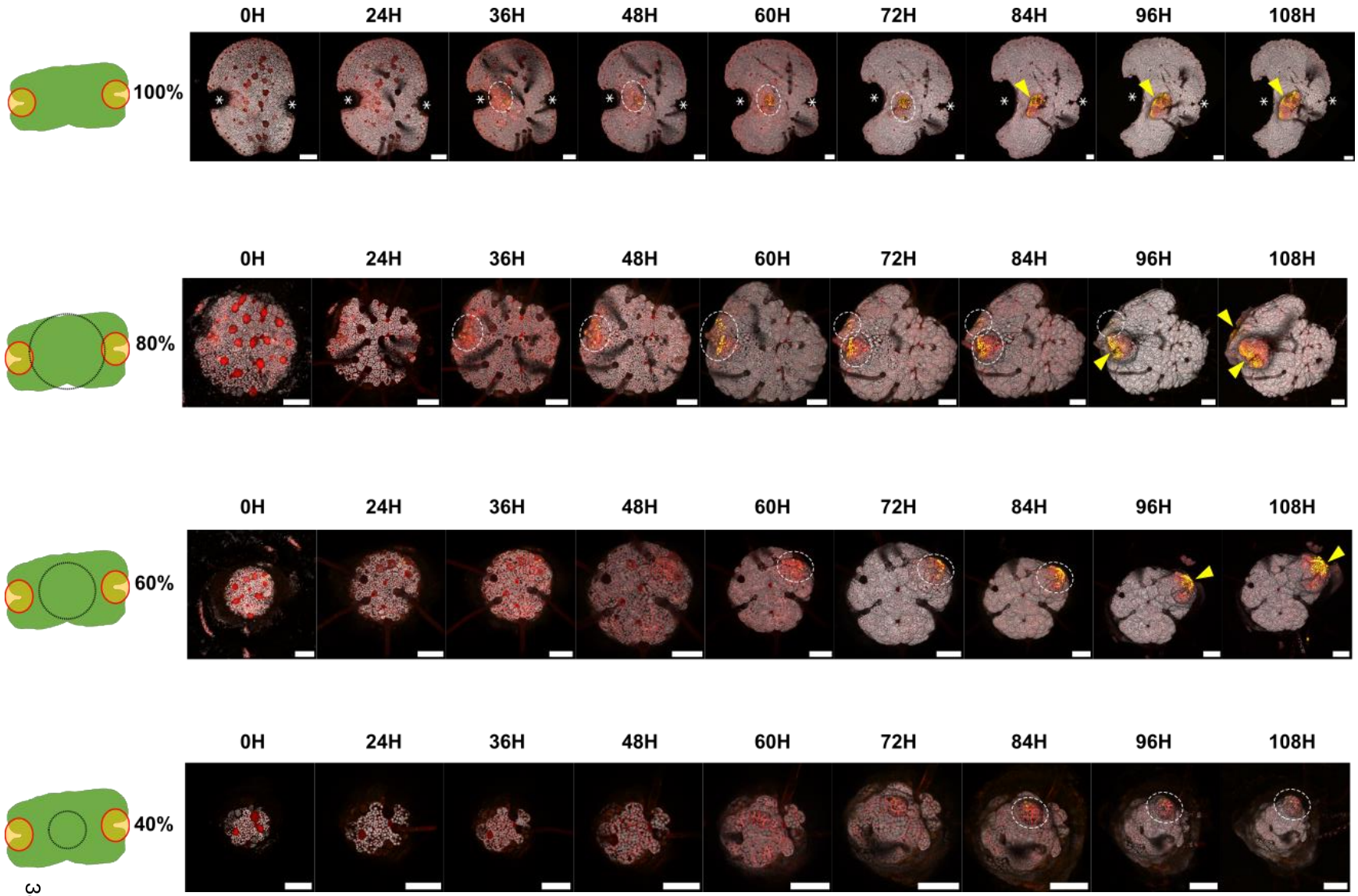
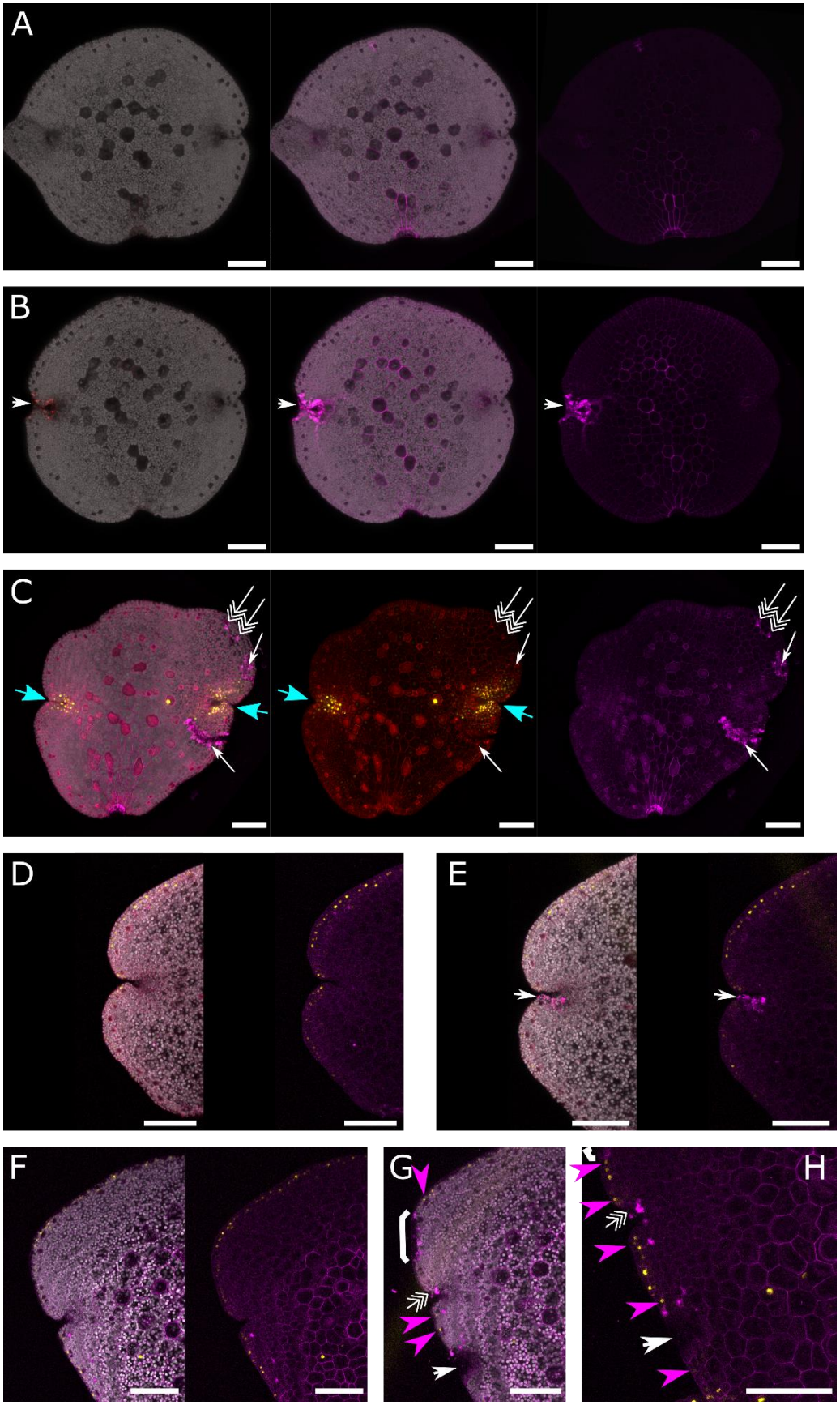

0H
24H
36H
48H
60H
72H
84H
96H
108H
100%
80%
60%
40%

Fig. S8. An example time course showing that meristem regeneration proceeds faster in larger gemma fragments. On the far left of each row is a schematic of the ablation pattern used, with orange bounded by red denoting excised tissue in the apical notch removal step (see Methods). The black circle shows the laser ablation circle trace used, with the circle diameter calculated as a percentage of the original notch-notch distance. Only the gemma fragment within the circle trace was retained, with all tissue outside of this destroyed. The emergence of localized patches of cell division, as defined by new, dense regions of mVenus marker signal, is indicated by dashed circles. Full meristem regeneration, as defined by the appearance of a notch within a region of dense mVenus marker signal, is indicated by yellow arrows. These definitions were used to generate the data shown in Fig. 5. Both the emergence of localized patches of cell division and full meristem regeneration occurred faster in larger fragments versus smaller fragments. This suggests that having a larger pool of cells to act as auxin sinks for the emerging auxin sources (i.e. new meristems) allowed for faster progression through the different phases of regeneration. Gemmae used were from the enhancer trap notch/meristem marker line ET239-P125. Time indicated is in hours after ablation. Scale bars= 100μm.


A
B
C
D
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F
G
H

Fig. S9. Propidium iodide staining shows that laser ablation kills targeted cells without causing widespread cell death or stress in the gemma. Propidium iodide (PI) staining is shown in magenta. PI stains cell walls but does not penetrate the cell membranes of living cells, however upon cell death it goes into the remains of the cell, staining the nucleus with fluorescence emission in the mScarlet channel wavelengths. (A) shows an intact WT gemma, with no nuclear PI staining. (B) shows a WT gemma whose apical notch was subjected to laser ablation. Targeted cells have nuclear and intracellular PI staining (white arrows), demonstrating that this treatment effectively killed cells in the Marchantia gemma. The damage (also indicated by loss of chlorophyll autofluorescence and mechanical disruption) was restricted to the area targeted by the laser, with neighbouring cells and the wider gemma left intact. (C) shows a gemma from an enhancer trap line (ET239-P14) following various laser ablation targeting treatments. Regions targeted for tissue excision (white arrows) featured multiple other obvious signs of cell damage (loss of chlorophyll autofluorescence, membrane destruction and mechanical disruption) and there were no signs of widespread cell death or stress across the rest of the gemma. Ablation targeting single cells (triple white arrows) only killed those cells. Laser ablation treatment did not have off-target effects on apical notch/meristem mVenus marker signal (blue arrows). (D) shows a gemma region before ablation; (E) shows the same region afterwards. The lower side of the apical notch had been ablated (white arrows in (E)) with no damage caused to the other side of the apical notch. (F) shows a gemma region before ablation, (G) shows the same region after ablation and (H) is a close-up of the same post-ablation gemma. The ablation patterns used were a row of cells at the gemma edge (white brackets), targeting single cells (triple white arrows) and tissue excision (white arrows). Only the targeted areas showed cell death, with agreement between PI staining, loss of chlorophyll autofluorescence and mechanical disruption. Neighbouring cells were unaffected, and enhancer trap marker signal continued unaffected (yellow arrows). (D)-(H) shows gemmae from the enhancer trap line ET239-P64. (A) and (B) show, from left to right, the chlorophyll autofluorescence and mScarlet emssion channels overlaid; chlorophyll autofluorescence and PI emission channels overlaid; PI emission channel only. (C) shows, from left to right, chlorophyll autofluorescence, mScarlet, PI emission and mVenus channels overlaid; mScarlet and mVenus channels overlaid; PI emission channel only. (D)-(G) shows, from left to right, chlorophyll autofluorescence, PI emission and mVenus channels overlaid; PI emssion and mVenus channels overlaid. (H) shows PI emssion and mVenus channels overlaid. Scale bars= 100μm.

Table S1. Excitation and collection wavelengths and settings used in confocal microscope imaging

Fluorophore	Excitation Wavelengths (nm)	Hybrid Detector Collection Wavelength Tuning (nm)	Laser Intensity	Gain
mVenus	515 (White Light Laser)	522-541	60%	60
mScarlet	569 (White Light Laser)	589-625	40%	50
Chlorophyll	405 (Diode)	670-701	6%	20
Propidium Iodide	488, 535 (White Light Laser)	592 - 650	15%, 10%	40

Supporting Information Text

Transgenic plant lines used for each figure. ET numbers (ET239-PXXX) refer to the Marchantia enhancer trap screening project (10)

Fig. 1 D: L2_239-CsA (ET239-P64) F: L2_239-CsA (ET239-P153)
Fig. 2 L2_239-CsA (A: ET239-P21 B: ET239-P21 C: ET239-P153 D: ET239-P21 E: ET239-P21)
Fig. 3 L2_239-CsA (ET239-P125)
Fig. 4 L2_239-CsA (B: ET239-P153 E: ET239-P153 H: ET239-P125) C, F, I: L2_268-CsA

Fig. S1 L2_239-CsA (A, B: ET239-P21 C,D: ET239-P33 E,F: ET239-P125 G,H: ET239-P153)
Fig. S2 L2_239-CsA (ET239-P153)
Fig. S4 L2_239-CsA (A:ET239-P125 B: ET239-P21 C: ET239-P21 D: ET239-P153 E: ET239-P21 F: ET239-P21)
Fig. S5 L2_239-CsA (ET239-P125)
Fig. S6 L2_239-CsA (B-E: ET239-P125 G-J, L-O: ET239-P153)
Fig. S7 L2_239-CsA (ET239-P21)
Fig. S8 L2_239-CsA (ET239-P125)
Fig. S9 A-F: Wild Type (Cam accession) G-I: L2_239-CsA (ET239-P14) J-Q: L2_239-CsA (ET239-P64)

Dataset S1 (separate file). Annotated sequence description of the plasmids used to generate the transgenic lines used in the experiments: L2_239-CsA (16,019 bp) and L2_268-CsA (19,174 bp). The sequences and annotations are provided in Genbank format.