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An Fgf/*Gremlin* Inhibitory Feedback Loop Triggers Termination of Limb Bud Outgrowth

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Abstract

During organ formation and regeneration a proper balance between promoting and restricting growth is critical to achieve stereotypical size. Limb bud outgrowth is driven by signals in a positive feedback loop involving fibroblast growth factor (Fgf) genes, *sonic hedgehog* (*Shh*) and *Gremlin1* (*Grem1*)[1]. Precise termination of these signals is essential to restrict limb bud size[2–4]. The current model predicts a sequence of signal termination consistent with that in chick limb buds[4]. Our finding that the sequence in mouse limb buds is different led us to explore alternative mechanisms. By analyzing compound mouse mutants defective in genes comprising the positive loop, we uncovered genetic evidence that FGF signaling can repress *Grem1* expression, revealing a novel Fgf/*Grem1* inhibitory loop. This repression occurs in both mouse and chick limb buds, and is dependent on high FGF activity. These data support a mechanism where the positive Fgf/*Shh* loop drives outgrowth and an increase in FGF signaling, which triggers the Fgf/*Grem1* inhibitory loop. The inhibitory loop then operates to terminate outgrowth signals in the order observed in either mouse or chick limb buds. Our study unveils the concept of a self-promoting and self-terminating circuit that may be used to attain proper tissue size in a broad spectrum of developmental and regenerative settings.

List of key genes

Fibroblast growth factor (Fgf); *Fgf4*; *Fgf8*; Fgf receptor (Fgfr); *Fgfr1*; *Fgfr2*; sonic hedgehog (*Shh*); gremlin 1 (*Grem1*); bone morphogenetic protein 4 (*Bmp4*); *Spry4*; *Msx2*; Brachyury (*T*); *Prx1*

Several models have recently been formulated to explain the control of appendage size[5–8]. The models focus on how a signal in constant supply is translated into a threshold of growth capability. Evidence from vertebrate limb development suggests that precise termination of growth signals is a key mechanism that restricts limb bud size[2–4]. These signals include Fgfs (*Fgf4*, *Fgf8*, *Fgf9* and *Fgf17*) expressed in the Apical Ectodermal Ridge (AER and AER-Fgfs), and *Shh* and *Grem1* expressed in the underlying mesenchyme. They function in a transcriptional feedback loop (Fgf/*Shh* loop) to induce and sustain each other's expression[1, 9–11].

The current model for breakdown of the Fgf/*Shh* loop is based on the observation that current and former *Shh*-expressing cells (*Shh*-lineage cells) are unable to express *Grem1* in response to SHH induction[4]. Expansion of the *Shh*-lineage would lead to cessation of *Grem1* expression

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Figure 4 of the main text summarizes the findings of this paper.

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followed by that of *Fgf4* and then *Shh*[4]. This sequence of signal termination is consistent with that observed in chick. However, we found that in mouse limb buds, *Fgf4* expression ceases first, followed by *Shh* and then *Grem1* (Fig. 1a–e). To identify alternative mechanisms of signal termination, we investigated the regulation of *Fgf4*, the first gene of the loop that ceases to be expressed in mouse limb buds. Although *Fgf4* itself is not essential for limb development[9], it is regulated by essential genes, including *Shh* and *Grem1*[10,12,13]. Furthermore, termination of *Fgf4* expression coincides with a drop in collective AER-FGF activity[14] (Fig. 1f–i). Therefore the extinction of *Fgf4* expression serves as readout for the trigger that breaks down the Fgf/*Shh* loop.

Fgf4 expression is severely reduced in *Shh* mutant and absent in *Grem1* mutant limb buds, but expanded and prolonged in *Fgf8* AER-knockout (*Fgf8-KO*) forelimb buds[10–13,15,16]. We investigated whether these regulators act genetically upstream or downstream of each other to control *Fgf4* expression. To address if *Fgf8* represses *Fgf4* expression by inhibiting *Shh* maintenance of *Fgf4*, we inactivated both *Fgf8* and *Shh* in the limb buds by introducing a null allele of *Shh*[17] into the *Fgf8-KO* background[15] (Msx2cre;$Fgf8^{fl/fl}$;$Shh^{-/-}$ mutant, or *Fgf8;Shh-DKO* for double knockout). In *Fgf8;Shh-DKO* forelimb and hindlimb buds, *Fgf4* is detected in an expanded pattern in the entire AER (Fig. 1j, k and data not shown), demonstrating that *Fgf8* repression of *Fgf4* expression is genetically downstream of *Shh*.

Grem1 functions downstream of *Shh* to induce *Fgf4* expression[10,11]. To address if *Fgf8* represses *Fgf4* expression by inhibiting *Grem1*, we inactivated both *Fgf8* and *Grem1* in the limb buds by introducing a null allele of *Grem1*[13] into the *Fgf8-KO* background (Msx2cre;$Fgf8^{fl/fl}$;$Grem1^{-/-}$ mutant, or *Fgf8;Grem-DKO*). In *Fgf8;Grem-DKO* limb buds, *Fgf4* is no longer maintained, even though the AER is present (Fig. 1l–n). With AER-Fgf expression severely compromised, all limb skeletal elements are absent (Fig. 1o–r), similar to the phenotype in *Fgf4* and *Fgf8* double mutant limbs[18]. This loss of *Fgf4* expression in *Fgf8;Grem-DKO* limb buds demonstrates that *Fgf8* repression of *Fgf4* is dependent on *Grem1*.

To understand the mechanism of this dependence, we investigated whether *Fgf8* represses *Grem1* expression. Consistent with this possibility, the *Grem1* domain is closer to the AER than normal in *Fgf8-KO* limb buds (Fig. 2a,b). As all AER-FGFs perform similar roles in limb bud outgrowth[19], we tested a more general hypothesis that collective AER-FGF signaling could repress *Grem1* expression. In support of this, the *Grem1* expression domain is closer to the AER in various other Fgf and Fgf receptor (Fgfr) mutants (Supplementary Fig. 1). One caveat is that these mutant limb buds are smaller than normal, raising the possibility that the *Grem1* domain is closer to the AER due to reduction of the distal mesenchyme. To test FGF repression of *Grem1* more rigorously, we inactivated *Fgfr1* and *Fgfr2* in a small portion of the limb bud mesenchyme (Shh^{cre};$Fgfr1^{co/co}$;$Fgfr2^{c/c}$ mutant, or *Fgfr1;r2-DKO*)[20–22]. We found that though FGF signaling is severely disrupted in Fgfr-inactivated cells, *Fgfr1;r2-DKO* limb buds exhibit normal size, shape, and cell survival at E11.5 (Fig. 2c,d and data not shown). In this setting, *Grem1* is ectopically expressed within Fgfr-inactivated domain (Fig. 2e–g, Supplementary Fig. 2). Our loss-of-function data complement a previous observation that FGF-soaked beads can inhibit *Grem1* expression in chick limb buds[23]. These data demonstrate that AER-FGF signaling is sufficient and necessary to repress *Grem1* expression in the distal mesenchyme.

High levels of exogenous Bone Morphogenetic Protein (BMP) have been shown to inhibit *Grem1* expression[23–25]. We found that *Bmp4* and *Bmp7* expression is reduced in the Fgfr-inactivated cells in *Fgfr1;r2-DKO* limb buds, raising the possibility that AER-FGFs repress *Grem1* by maintaining high BMP signaling (Fig. 2h,i and data not shown). However, inactivation of *Bmpr1a* with Shh^{cre} does not lead to ectopic *Grem1* expression (Fig. 2j,k),

suggesting that AER-FGF repression of *Grem1* is not mediated through BMPs. It remains possible that BMP signaling may be required to promote *Grem1* expression in parallel to FGF repression of *Grem1*[23–27] (Fig. 2l).

To investigate the threshold requirement for FGF repression of *Grem1*, we compared *Grem1* expression to changes in FGF signaling. In mouse limb buds, downregulation of *Grem1* in the distal mesenchyme correlates with progressively higher levels of FGF signaling as development proceeds (Fig. 3a–e). This result is consistent with that observed in chick limb buds[23,25]. These gene expression data led us to hypothesize that AER-FGF signaling represses *Grem1* in a dose-sensitive manner.

We tested this hypothesis in both mouse and chick limb buds. In chick, implantation of beads soaked in 1mg/ml of FGF2 leads to a clear repression of *Grem1* (n=4/6, Fig. 3i), consistent with previous observation[23]. This repression is not observed using beads soaked in 0.1mg/ml of FGF2 (n=0/7), even though *Spry2* upregulation is detected adjacent to the beads, confirming FGF activity (data not shown). In mouse, ectopic *Grem1* expression is more intense in *Fgfr1;r2-DKO* limb buds compared to $Shh^{cre};Fgfr1^{co/co}$ (*Fgfr1-KO*) limb buds (Fig. 3j–n). As there is less residual FGF signaling in *Fgfr1;r2-DKO* limb buds than that in *Fgfr1-KO* limb buds (based on expression of FGF readouts, data not shown), lower FGF signaling correlates with less *Grem1* repression. Thus data from both chick and mouse limb buds support the scenario that, during limb bud outgrowth, a progressive increase in AER-FGF level leads to increasing repression of *Grem1* in the distal mesenchyme.

To return to our question regarding the mechanism that abolishes *Fgf4* expression and triggers Fgf/*Shh* loop termination, we found that *Fgf4* expression is prolonged in *Fgfr1;r2-DKO* forelimb buds at E11.75 (Fig. 3o,p), likely as a result of ectopic *Grem1* expression[11–13,23]. These data demonstrate that FGF repression of *Grem1* plays a critical role in triggering the termination of limb bud outgrowth signals.

The finding that AER-FGF signaling can repress *Grem1* expression reveals an inhibitory feedback loop (Fgf/*Grem1* loop) that is interconnected with the existing Fgf/*Shh* positive feedback loop (Fig. 4a). The dosage dependency of this repression led us to propose a model (Fig. 4b) whereby positive and inhibitory feedback loops are coordinated first to promote (in phase I, with Fgf/*Shh* positive loop only) and later to terminate limb bud outgrowth (in phase II, with the induction of Fgf/*Grem1* inhibitory loop). In a wild-type limb bud in phase I (e.g. ~E9.5–E10.5 in mouse forelimb bud, ~stage18–23 in chick wing bud), we hypothesize that in phase I, AER-FGF concentration is too low to efficiently repress *Grem1* (Fig. 3c,d,g,i). Instead, AER-FGFs act through *Shh* and BMPs to upregulate *Grem1*[10,24,25]. As a result, *Grem1* is expressed in the distal mesenchyme abutting the AER (Fig. 3a) and efficiently promotes AER-Fgf expression [3,26,28,29]. In phase I, the positive Fgf/*Shh* loop induces and sustains limb outgrowth signals leading to a progressive increase in collective AER-FGFs entering phase II (Fig. 3c–e).

We hypothesize that the transition to phase II occurs when AER-FGF signaling surpasses the threshold needed for *Grem1* repression in the distal mesenchyme, triggering the *Fgf*/*Grem1* inhibitory loop (Fig. 4b, e.g. ~E10.5–E12 in mouse forelimb bud, ~stage 23–27 in chick wing bud). This repression establishes a *Grem1*-negative domain separating *Grem1*-expressing cells and the AER (Fig. 2a). As development proceeds, the *Grem1*-negative domain expands both distally and posteriorly due to mesenchymal growth. We postulate that this expansion would trigger different rate-limiting steps in mouse versus chick limb buds, leading to distinct sequences of signal termination. In a mouse limb bud, the size of the *Grem1*-negative domain would first exceed the distal range of GREM1 protein diffusion, leading to downregulation of collective AER-FGFs followed by loss of *Shh* and then *Grem1* expression (Fig. 4b, end of

phase II). Loss of *Grem1* expression would mark the beginning of AER degeneration and gradual extinction of *Fgf8* expression[12,13]. Conversely in a chick limb bud, the size of the *Grem1*-negative domain would first exceed the anterior range of SHH diffusion, leading to loss of *Grem1* expression followed by extinction/reduction of different AER-Fgfs and then termination of *Shh*. Thus, this model can explain the sequence of signal abrogation in both mouse and chick. We further postulate that in a wider spectrum of divergent species, parameters such as signal diffusion range, threshold requirement of signaling activity and extent of mesenchyme expansion dictate the timing of outgrowth signal termination.

There are two key differences between our model and the existing model of signal termination[4] (*Shh*-lineage model). First, the *Shh*-lineage model only accounts for *Grem1* repression in posterior mesenchyme. Our model explains *Grem1* repression in both the posterior and distal mesenchyme, which accommodates the sequence of signal termination in both mouse and chick limb buds. Second, the molecular mechanisms at the core of the two models are distinct. In the *Shh*-lineage model, the factor responsible for cell-autonomous repression of *Grem1* in *Shh*-lineage cells has not been identified. Our finding that *Fgfr* inactivation allows *Grem1* expression in *Shh*-lineage cells (Fig. 2f,g, Fig. 3k–n) suggests that maintenance of FGF signaling is essential for *Grem1* repression in this lineage. In our model, signal termination relies on FGF repression of *Grem1* expression. The finding that an FGF bead placed in the anterior chick limb bud downregulates *Grem1* expression[23] (Fig. 3i) indicates that FGF repression of *Grem1* can occur independent of the *Shh*-lineage influence.

In this study, we identified an inhibitory Fgf/*Grem1* feedback loop that operates in both mouse and chick limb buds. We propose a model whereby the known positive Fgf/*Shh* feedback loop acts to increase AER-FGF concentration, triggering the inhibitory loop, which in turn leads to extinction of outgrowth signals. These interconnected positive and inhibitory loops direct a limb outgrowth program that once initiated, can propagate and self-terminate.

Supplementary Material

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Acknowledgments

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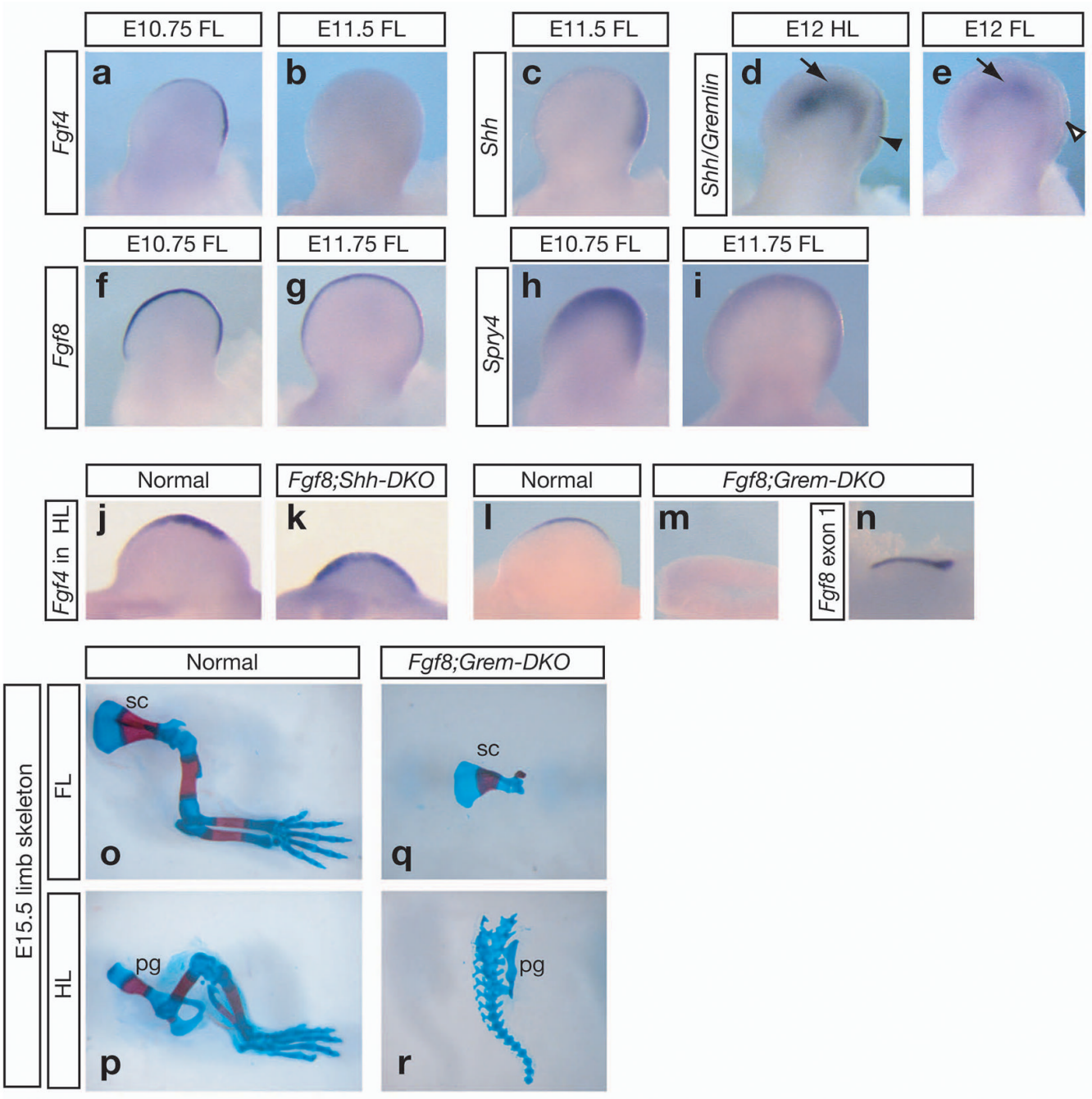

Figure 1. *Fgf8* repression of *Fgf4* expression is dependent on *Grem1* but not *Shh*

(a–n) Gene expression in mouse forelimb (FL) or hindlimb (HL) buds. **(a–i)** In wild-type mouse limb buds, *Fgf4* expression terminates first, followed by *Shh* and then *Grem1*. In **d** and **e**, a combination of RNA probes is used to detect non-overlapping patterns of *Shh* (arrowhead) and *Grem1* (arrow) expression. Both genes are expressed in the E12 hindlimb bud, which is at an earlier developmental stage than the E12 forelimb bud from the same embryo, where only *Grem1* is expressed(n=4). Downregulation of *Spry4* expression at E11.75 compared to E10.75 reflects decreased AER-FGF activity[14], consistent with loss of *Fgf4* and reduced *Fgf8* expression. **(j–m)** In E10.5 hindlimb buds, *Fgf4* expression is detected in the posterior two-thirds of the AER in normal, expanded through the entire AER in the *Fgf8;Shh-DKO* mutant

and absent in *Fgf8;Grem1-DKO* mutant. (**n**) Detection of the remaining exon 1 of the truncated *Fgf8* mRNA indicates that the AER is present. (**o–r**) No forelimb or hindlimb elements are observed in *Fgf8;Grem-DKO* skeletons. *Fgf8;Shh-DKO* embryos were generated by mating *Msx2cre;Fgf8*$^{del/flox}$*;Shh*$^{+/-}$ males to *Fgf8*$^{flox/flox}$*;Shh*$^{+/-}$ females[15,17]. *Fgf8;Grem-DKO* embryos were generated by crossing *Msx2cre;Fgf8*$^{del/+}$*;Grem1*$^{+/-}$ males to *Fgf8*$^{flox/flox}$*;Grem1*$^{+/-}$ females[13,15]. sc, scapula; pg, pelvic girdle.

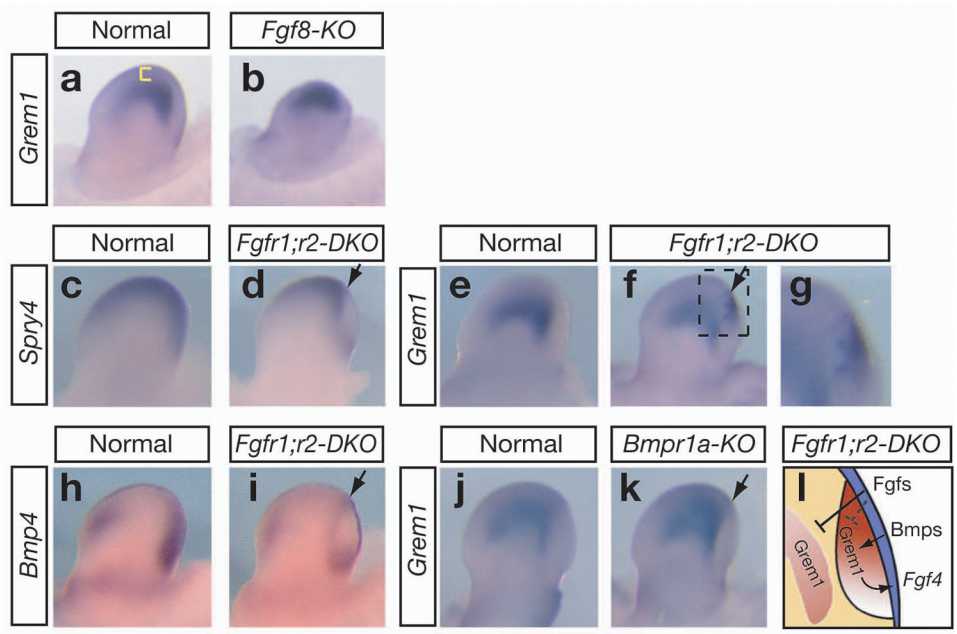

Figure 2. FGF signaling represses *Grem1* expression

(**a–k**) Gene expression in mouse forelimb buds at (**a,b**) E10.75, (**c–i**) E11 and (**j,k**) E11.5. (**a**) The yellow bracket indicates distance between AER and high *Grem1* expression. (**c,d**) Reduced *Spry4* expression in the posterior mesenchyme delineates Fgfr-inactivated domain. Arrows in **d**, **f**, **i**, **k** indicate anterior boundary of Shh^{cre}-mediated receptor inactivation domain. (**e–g**) *Grem1* is ectopically expressed in the distal portion of the *Fgfr*-inactivated domain. Limb buds shown in **d** and **f** are contralateral limb buds from the same embryo. Boxed region in **f** is magnified in **g**. (**h,i**) *Bmp4* is reduced in Fgfr-inactivated domain, but is present in the overlying AER. **(j,k)** No ectopic *Grem1* expression is detected in $Shh^{cre};Bmpr1a^{fl/fl}$ (*Bmpr1a-KO*) limb buds. **(l)** A diagram depicting gene expression regulation within the Fgfr-inactivated domain in *Fgfr1;r2- DKO* limb buds as shown in **g**. Bmps from the AER may be required to promote ectopic *Grem1*[23–27], leading to higher *Grem1* in the distal portion of the Fgfr inactivated domain as shown in Fig. 2g, Fig. 3l,n. *Fgfr1;r2-DKO* embryos were generated by mating $Shh^{cre};Fgfr1^{co/co};Fgfr2^{c/+}$ males to $Fgfr1^{co/co};Fgfr2^{c/+}$ females[20–22]. Bmpr1a-DKO embryos were generated by mating $Shh^{cre};Bmpr1a^{fl/+}$ males to $Bmpr1a^{fl/fl}$ females[21,30].

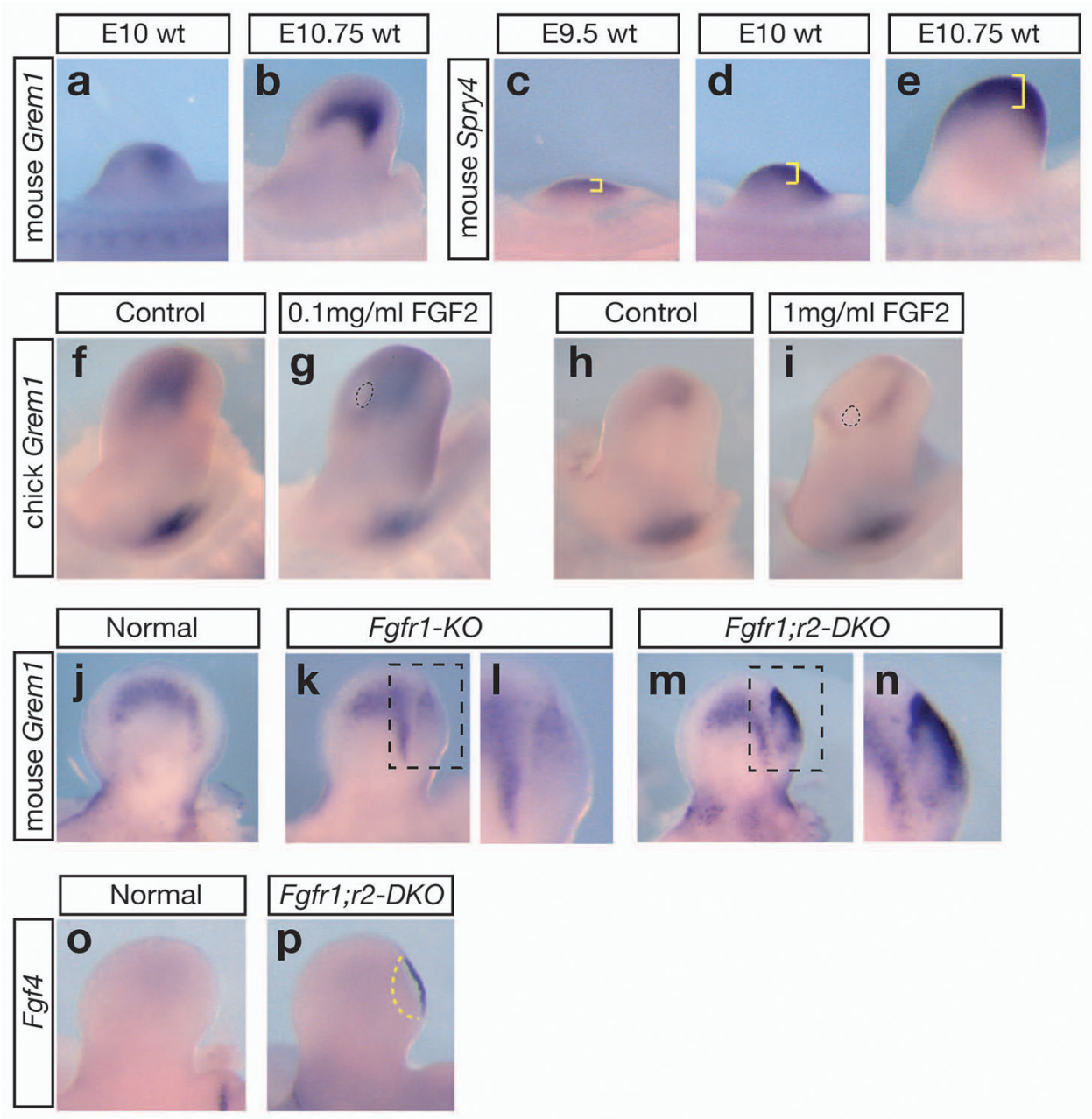

Figure 3. AER-FGF repression of *Grem1* expression is dose-sensitive

(a–e) Correlation between *Grem1* repression in the distal mesenchyme and increased AER-FGF signaling (yellow brackets in c–e)[14]. **(f–i)** Beads (circle) soaked in 1mg/ml FGF2 suppress *Grem1* expression distal to the bead, possibly working in combination with FGFs expressed from the AER (n=4/6). No *Grem1* suppression is observed with 0.1mg/ml FGF2 (n=7). Beads were implanted in stage 21 limb buds and gene expression was assayed after 12 hours of incubation. **(j–n)** While ectopic *Grem1* expression is more intense in E11.5 *Fgfr1;r2-DKO* limb buds than in *Fgfr1-DKO* limb buds, *Grem1* expression outside of the Fgfr-inactivated domain remains comparable. Boxed regions in **k,m** are magnified in **l,n**, respectively. **(o,p)** Though absent in E11.75 normal limb bud, *Fgf4* expression persists in the posterior AER overlying the Fgfr-inactivated domain in *Fgfr1;r2-DKO* limb buds (delineated by yellow dashed line).

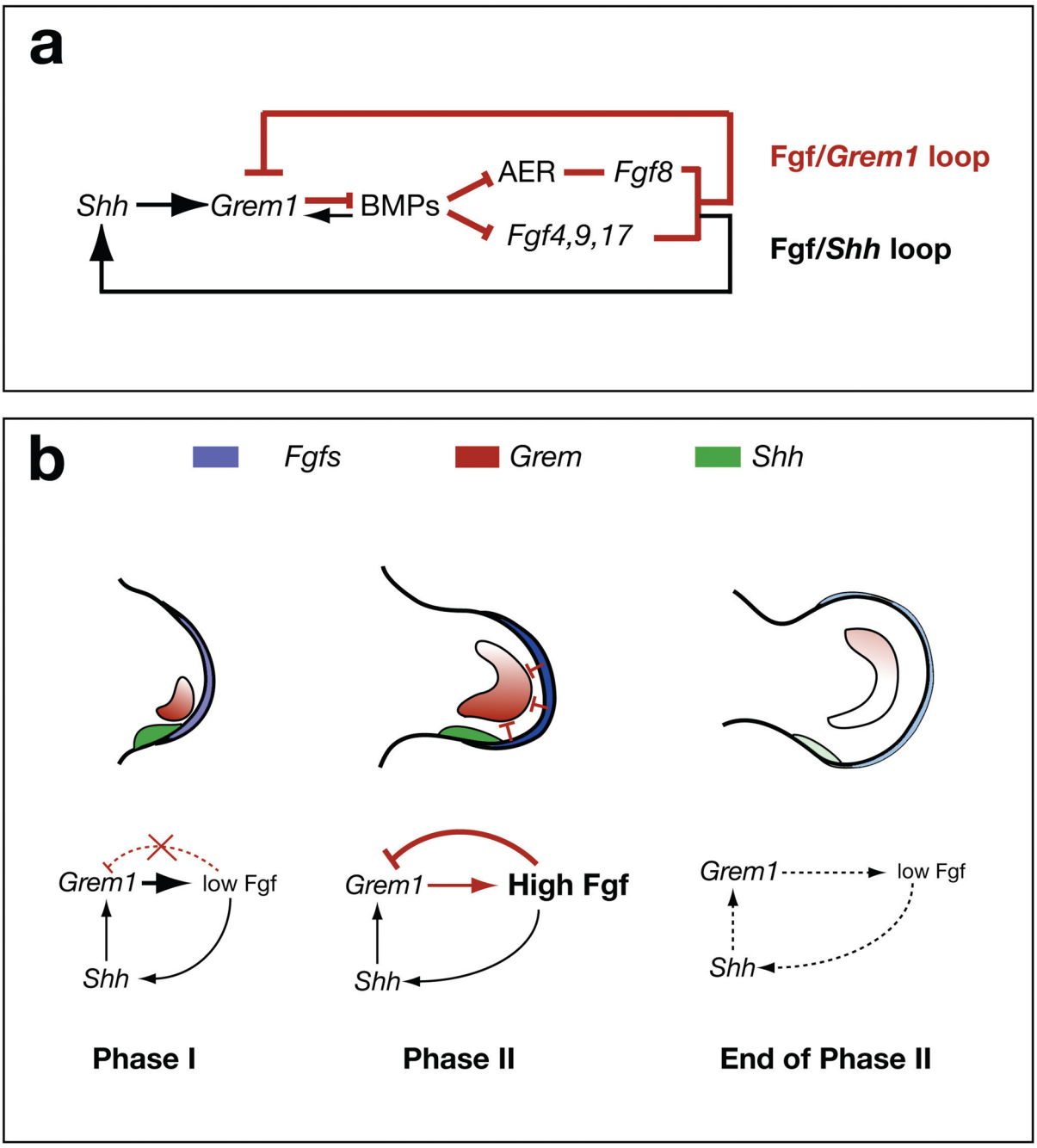

Figure 4. A model describing a self-promoting and self-terminating mechanism to control limb bud outgrowth signals

(**a**) A schematic of the inhibitory loop (outlined in red) in relation to the existing positive loop. Arrows indicate activation, while "T" lines indicate inhibition. BMP regulation of AER architecture indirectly affects *Fgf8* expression[12,13]. *Grem1* is also positively regulated by BMP signaling[11,24,25,27]. (**b**) A model explaining how the two loops are utilized to first promote (phase I) and then terminate (phase II) signals. Dashed lines represent diminishing regulation while dashed line with "X" emphasizes absence of regulation. In phase I, the positive regulatory loop operates to increase all signals. Transition to phase II occurs when AER-FGFs reach a level that confers efficient *Grem1* repression (represented by "T" in both distal and posterior

mesenchyme). Together with mesenchymal growth, the *Grem1*-negative domain expands. Increasing distance between *Grem1*-expressing cells and *Fgf* or *Shh*-expressing cells leads to inability of signals to maintain one another at the end of phase II.