Developmental Cell *14*

Supplemental Data

Uncoupling Sonic Hedgehog Control of Pattern and Expansion of the Developing Limb Bud

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Table S1. Distributions of Digit Reductions (Loss or Attenuation) Following Tamoxifen (Tam.) Injection at Different Times

Total # of digits formed:	**5**	**4.5**	**4**	**3.5**	**3**	**2**	**1**
FL (total n = 100):	22	6	35	15	8	12	2
HL (total n = 100):	0	5	34	17	12	3	29
digit loss phenotype:	**normal**	**----mild----**		**---moderate---**		**----severe----**	
*Time range Tam. injection for **FL** phenotypes:	**E10.25-E10.75**			**E9.5-E10.25**		**~E9.5**	
*Time range Tam. injection for **HL** phenotypes:	**E10.25-E11**			**E9.75-E10.25**		**E9.5-E9.75**	

* Forelimbs (FL) and hindlimbs (HL) are grouped separately into the different phenotype classes and have different ranges of injection times for which those phenotypes were observed (as indicated), because HL development lags about 12 hours behind FL. Consequently, HL phenotypes were usually more severe than FL in the same embryo.

Table S2. Frequencies of Different Digits Lost as 1st, 2nd, and 3rd, Inferred from Remaining Digits and Presence of Attenuated Forms

	First digit lost [3.5 to 4.5 digits present]:			**Second digit lost** [3 to 3.5 digits present]:			**Last two digits remaining** [2 digits present]:		
	d3	**non-d3**	**?ID**	**d5**	**non-d5**	**?ID**	**d1+d2**	**d1+d4**	**?ID**
FL:	**42/56**	**4/56**	**10/56**	**22/23**	**0/23**	**1/23**	**2/12**	**7/12**	**3/12**
HL:	**51/56**	**0/56**	**5/56**	**26/29**	**0/29**	**3/29**	**0/3**	**2/3**	**1/3**

Forelimbs (FL), hindlimbs (HL), uncertain identity (?ID), numerators indicate number involving indicated digit type (eg. d3) over denominator of total specimens in that category (eg. FL with 3.5-4.5 digits).

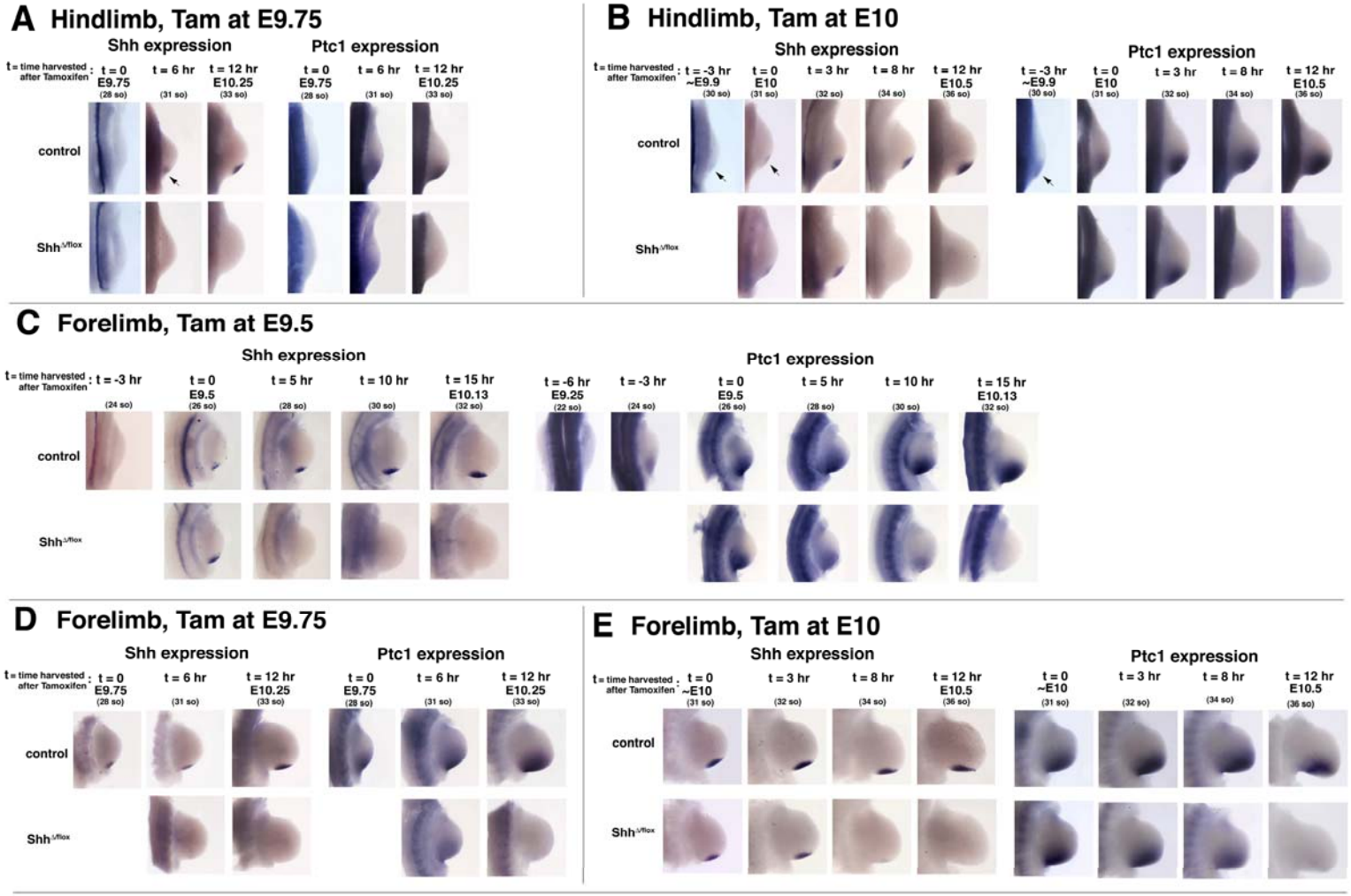

Figure S1. Time Course Analysis of *Shh* and *Ptc1* Expression Following Tamoxifen-Induced Timed Removal of the *Shh-flox* Allele

Embryos were harvested at different times (t) following single dose tamoxifen (Tam) treatment and somite numbers (so) were counted. In all cases assayed at that time point, *Shh* expression was completely lost within 5-6 hrs after Tam injection, and *Ptc1* reporting of Shh activity was lost within ~12 hrs after Tam injection (A, B). Untreated control embryos were evaluated at closely spaced age intervals between E9.25-E10 to determine the onset of Shh activity more precisely. *Shh* was first detectable at 30 so (~E9.9, arrows in A,B) in hindlimb (HL) and 25-26 so (~E9.5, C) in forelimb (FL), and *Ptc1* was already detectable at the same time or slightly earlier (30 so in HL--arrows in B; 24 so in FL--E9.4 in C). In HL, the total duration of Shh activity extended for 9 hrs when Tam was given at E9.75 (A) or for 15hrs when Tam was given at E10 (B), indicating that transient Shh signaling was sufficient to pattern the posterior digit 4 (see text). (C-E) In FL, the total duration of Shh activity was estimated to extend for ~15 hrs when Tam given at E9.5 (C), 21 hrs when Tam was given at E9.75 (D) or 27 hrs when Tam was given at E10 (E); taking E9.9 and E9.4 as the times of first appearance of Shh activity in HL and FL respectively. Control embryos were $Shh^{+/flox}$;*Cre*+ and $Shh^{\Delta/flox}$;*Cre*- siblings.

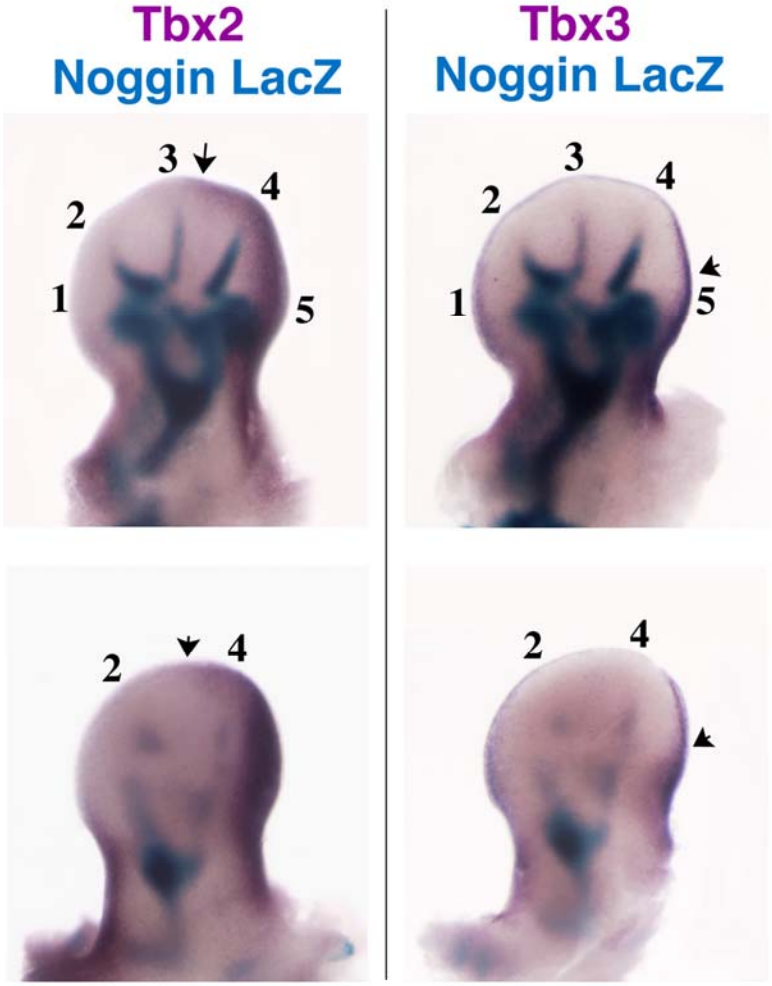

Figure S2. Expression of *Tbx2* and *Tbx3* at Very Early Digit Condensation Stages

E11.25-E11.75 limb buds were analyzed for *Tbx2* or *Tbx3* expression to assess the identity of the earliest forming condensations (visualized by Noggin-LacZ (*Noggin*$^{LacZ/+}$) activity). Once all condensations had appeared (upper panels), mesenchymal *Tbx2* expression extended to the posterior side of digit 3 and surrounded digit 4 and 5 condensations, whereas mesenchymal *Tbx3* expression surrounded only the digit 5 condensation (arrows in upper panels), as previously reported (Suzuki et al, Dev Cell 6, 43-53, 2004). Note that *Tbx3* was also expressed in the AER (Gibson-Brown et al, Mech Dev 56, 93-101, 1996). Earlier, when only two condensations were present, *Tbx2* expression surrounded the posterior condensation, identifying it as digit 4 and/or digit 5, but did not extend (arrow) near to the anterior condensation, identifying it as digit 2. In contrast, *Tbx3* expression was more posteriorly restricted (arrow) and did not extend to the posterior condensation, indicating that it represented digit 4 rather than indeterminate digit 4-5 precursors.

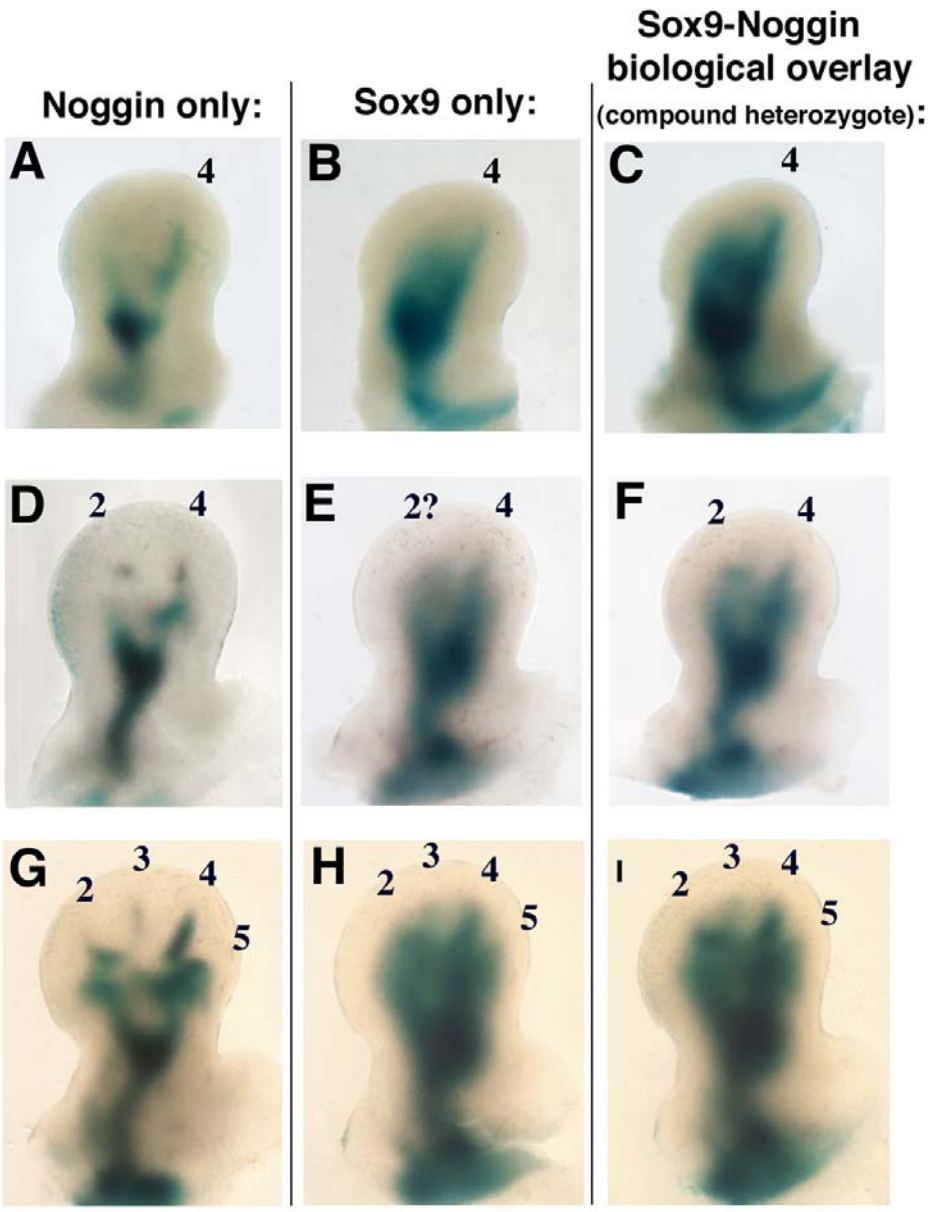

Figure S3. Order of Formation of Normal Digit Condensations Compared using Noggin-LacZ and Sox9-LacZ Expression to Visualize Early Condensations

LacZ activity was assayed in E11.25-E11.75 limb buds from *Noggin-LacZ* (*Noggin*$^{LacZ/+}$ A, D, G), *Sox9-3'UTR-LacZ* (*Sox9*$^{LacZ/+}$ B, E, H), and compound heterozygous *Noggin*$^{LacZ/+}$;*Sox9*$^{LacZ/+}$ (C, F, I) sibling embryos. Although Sox9 expression initiated weakly and almost synchronously in digit condensations, the digit 4 condensation clearly appeared first (B), followed by digit 2 (E), and then by the rest in rapid succession (H). Biological overlay of Noggin and Sox9 expression (in compound *Noggin*$^{LacZ/+}$;*Sox9*$^{LacZ/+}$ embryos, C, F, I) showed coincident staining of condensations, and displayed the same pattern as their single positive littermates (A, D, G, and B, E, H), confirming that both markers visualize the same subset of digits.

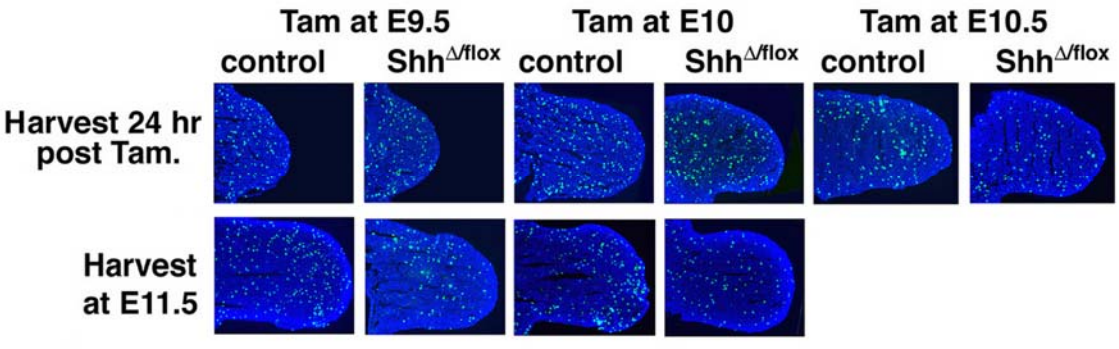

Figure S4. Analysis of Mitotic Rate Following Timed Removal of *Shh*
Mitoses were visualized with anti-pH3 antibody and DAPI nuclear counterstaining in hindlimb bud sections from $Shh^{\Delta/flox}$;*Cre*+ mutant and control sibling embryos ($Shh^{+/flox}$;*Cre*+ and $Shh^{\Delta/flox}$;*Cre*-) at the indicated times after Tamoxifen (Tam) injection. Several sections from each of 1-3 independent limb buds of each genotype for each time point were evaluated to determine the mitotic indices presented in Figure 4B.

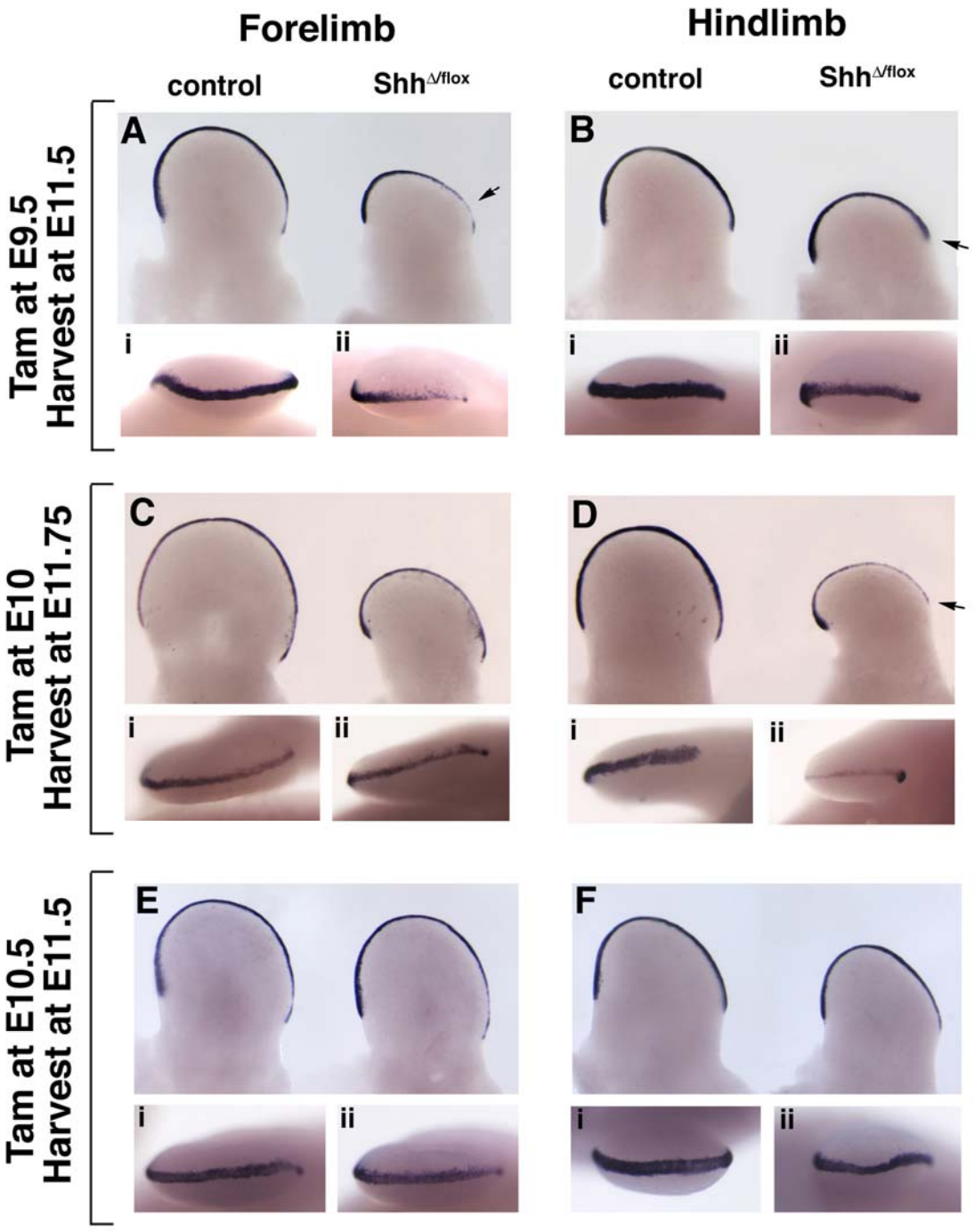

Figure S5. Effect of *Shh* Removal at Different Times on AER Maintenance
Shh$^{\Delta/flox}$;*Cre*+ and control sibling embryos (*Shh*$^{+/flox}$;*Cre*+ and *Shh*$^{\Delta/flox}$;*Cre*-) were harvested at E11.5-11.75, following Tamoxifen treatment (Tam) at the times indicated and analyzed for *Fgf8* expression. In all panels, the limb bud anterior side is to the right. The lower i, ii panels of distal limb bud on-edge views show the AER thickness. The AER was both decreased in thickness and in its anterior extent in *Shh* mutant embryos (eg. arrows). Sections also showed a modest decrease in AER ectodermal height following early *Shh* removal (data not shown). These effects were more pronounced at earlier times of *Shh* removal, and in hindlimb at a given time (which is ~12 hours delayed in development compared to forelimb).