Delineating a Conserved Genetic Cassette Promoting Outgrowth of Body Appendages

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Abstract

The acquisition of the external genitalia allowed mammals to cope with terrestrial-specific reproductive needs for internal fertilization, and thus it represents one of the most fundamental steps in evolution towards a life on land. How genitalia evolved remains obscure, and the key to understanding this process may lie in the developmental genetics that underpin the early establishment of the genital primordium, the genital tubercle (GT). Development of the GT is similar to that of the limb, which requires precise regulation from a distal signaling epithelium. However, whether outgrowth of the GT and limbs is mediated by common instructive signals remains unknown. In this study, we used comprehensive genetic approaches to interrogate the signaling cascade involved in GT formation in comparison with limb formation. We demonstrate that the Fgf ligand responsible for GT development is Fgf8 expressed in the cloacal endoderm. We further showed that forced Fgf8 expression can rescue limb and GT reduction in embryos deficient in Wnt signaling activity. Our studies show that the regulation of Fgf8 by the canonical Wnt signaling pathway is mediated in part by the transcription factor Sp8. Sp8 mutants elicit appendage defects mirroring Wnt and Fgf mutants, and abolishing Sp8 attenuates ectopic appendage development caused by a gain-of-function β-catenin mutation. These observations indicate that a conserved Wnt-Sp8-Fgf8 genetic cassette is employed by both appendages for promoting outgrowth, and suggest a deep homology shared by the limb and external genitalia.

Introduction

Development of the external genitalia is a crucial aspect of mammalian evolution that enables internal fertilization, a pivotal step towards land invasion. All therian mammals including metatherians develop external genitalia around their urogenital outlets. In mice, development of the embryonic anlage of external genitalia, the genital tubercle (GT), is identical in both sexes before androgen-mediated penile masculinization which occurs around embryonic day 16. The early androgen-independent phase of GT development is achieved through coordinated growth and patterning of cloacal endoderm-derived urethral epithelium (UE), mesoderm-derived para-cloacal mesenchyme (PCM) and the ventral ectoderm, which results in a cone-like structure with a ventral-medial positioned urethra surrounded by GT mesenchyme within an ectodermal epithelial capsule.

The development of the GT as an unpaired body appendage, is often compared to that of the paired-type appendages, the limbs [1,2]. Despite their anatomical and functional differences, the morphogenesis of both structures appears to involve similar genetic controls. Regulatory genes/pathways including canonical Wnt signaling [3,4], HH signaling [5–7], BMP signaling [8,9] and Hox genes [10–14] are essential for the development of both appendages. Some have suggested that the regulatory mechanisms common to both might be evolutionarily linked [11,15,16].

The first step in appendage outgrowth is the establishment of an independent proximodistal developmental axis apart from the main body axis. This process requires precise regulation from instructive signals, which often come from a distal signaling center. In addition to promoting and maintaining outgrowth, these signals also provide directional information that determines the orientation and shape of future structures. Moreover, genes required for subsequent patterning and differentiation are often regulated by the distal signaling center. For example, during limb development, the initiation and continuous outgrowth of the limb bud rely on growth factors secreted from a strip of ectodermal epithelium, termed the apical ectodermal ridge (AER), positioned at the distal edge of limb bud [Figure 1A and 1E]. Fibroblast Growth Factors (FGFs) are crucial signals provided by the AER, as FGF-soaked beads can replace the AER to induce limb outgrowth [17–20]. Furthermore, the AER FGF signals are obligatory to maintain the AER itself, and AER derived signals are necessary for outgrowth and shape of embryonic appendages [21].
Author Summary

Mammalian limbs and external genitalia are body appendages specialized for locomotion and internal fertilization, respectively. Despite their marked anatomical and functional differences, development of the limb and external genitalia appears to involve similar genetic controls, and some have suggested that regulatory mechanisms common to both might be evolutionarily linked. One essential aspect for appendage development is the establishment and maintenance of a separated proximodistal developmental axis apart from the main body axis, which is often instructed by a distal signaling epithelium. Herein, we adopted comprehensive mouse genetic approaches to investigate regulatory mechanisms underlying the distal signaling center in the limb and the GT, and uncovered a conserved genetic cassette that is utilized by both paired and unpaired appendages to establish a distal signaling center in the epithelium that mediates subsequent proximodistal outgrowth. Our results further suggested that the evolution of the external genital organ involved co-option of the same genetic program underpinning limb development.

Unlike limb, subsequent outgrowth of the GT has to accommodate a continuous extension of the cloacal endoderm, which forms the epithelial lining of the future urethral tube. This unique pattern of GT development suggests that the centrally located distal cloacal endoderm (later the distal urethral epithelium or dUE, marked in red in Figure 1B–1D, also shown by Fgf8 expression in situ in Figure 1F–1H) is a strategic place for GT outgrowth. Consistently, an earlier study demonstrated that Fgf8 expression is expressed in a strip of cells located at the distal-most part of the cloacal endoderm right below the ventral ectoderm (Figure 1B inset). Subsequent functional analyses revealed that physically removing Fgf8-expressing cells or application of neutralizing FGF8 antibody could abolish GT growth in organ culture, and this growth inhibition could be reversed by adding FGF8-soaked beads [24].

Along with these studies, we uncovered that activity of the canonical WNT-β-catenin pathway is restricted to the Fgf8-expressing distal cloacal endoderm and later the dUE [4]. We found that abolishing β-catenin (β-Cat) in the cloacal endoderm caused GT agenesis/reduction, whereas ectopic activation of the same pathway resulted in GT over-development. Interestingly, the site and level of WNT signaling activity positively correlated with Fgf8 expression and the extent of outgrowth in both limbs and GT. These findings illustrated a parallel between dUE and AER signaling during appendage outgrowth. However, the exact mechanisms and functional relevance for this WNT-Fgf8 regulation remain to be elucidated.

Recently, two independent studies reported a surprising observation that abolishing Fgf8 [25,26], or simultaneously abolishing Fgf4 and Fgf8 [26], in the cloacal endoderm does not affect GT outgrowth or GT-specific gene expression. These results questioned the relevance of FGF signals in external genitalia development and challenged the view that GT formation is organized and maintained by the dUE, which further suggested that the mechanisms underpinning limb and GT outgrowth are indeed divergent [2].

Herein, we adopted comprehensive genetic approaches to address the inductive signals in GT development, in comparison with that of the limb. We sought to define the role for FGF signaling and dUE-expressed Fgf8 in the GT, and explore mechanisms upstream of FGF activation in the dUE of the GT and AER of the limb. Results from our analyses revealed a remarkably conserved Wnt-Sp8-Fgf8 genetic circuitry that is crucial for proximodistal outgrowth of both paired- and unpaired-type appendages in mice.

Results

FGF Responsiveness Is Indispensable for GT Development

Previous studies demonstrated that inactivating one or two FGF ligands did not affect genital development [25,26]. We reasoned that the extensive genetic redundancy among FGF ligands might have undermined the power of these experiments. Therefore, we

Figure 1. Distal signaling epithelia in the outgrowth of body appendages. (A) The outgrowth of the limb is instructed by a specialized ectodermal epithelium, the AER (in red), positioned at the distal edge of the limb bud expanding across the anteroposterior axis. Inset shows a cross-section through the plane indicated by arrow. (B) The Fgf8-expressing GT signaling center dUE (in red) is positioned at the distal most part of the cloacal endoderm right below the ventral ectoderm (inset, coronal section through the plain indicated by arrow). (C, D) The dUE remains at the distal end of the urethral epithelium as the GT and the urethra undergo continuous proximodistal outgrowth. Endodermal cloaca was illustrated in yellow in B–D. (E–H) Fgf8 expression marks the AER (E) and dUE (F–H). bl, bladder; ls, lateral swellings; ds, dorsal swelling; ugs, urogenital sinus; ue, urethral epithelium; r, rectum; hg, hindgut; tg, tail gut; PCM, para-cloacal mesenchyme.

doi:10.1371/journal.pgen.1003231.g001
sought to re-evaluate the function of FGF signaling in GT development by conditionally abolishing FGF receptors. Fgfr1 and Fgfr2 are the only FGF receptors expressed in the developing GT [27]. Both Fgfr1 and Fgfr2IIc are expressed in the para-cloacal mesenchyme (PCM) during GT outgrowth [27], and Fgf2IIb is expressed in both the PCM and the urethral epithelium (UE) [27,28]. To abolish all FGF responsiveness in the developing GT, we employed an Msox2TakAPeO-Cre system which enables doxycycline (Dox)-inducible gene ablation in both the PCM and the UE [29,30]. Dox was given to pregnant females on embryonic day (E) 9.5 and E10.5 to induce Cre-mediated recombination of the Fgfr1 IIc [31] and Fgfr2 IIc [32] alleles. The phenotypes of Msox2TakAPeO-Cre Fgfr1 IIc Fgfr2 IIc mutant embryos [hereafter referred to as GT-Fgfr1 IIc double conditional knockouts (cKO), or dcKO] were analyzed by scanning electron microscopy (SEM).

GT-Fgfr1 IIc-2-dcKO genital tubercles were underdeveloped compared to their littermate controls at all stages examined starting from E11.5, when a clear tubercle structure could first be detected (Figure 2B). At E12.5, the dcKO GTs showed a clear deficiency in proximodistal outgrowth, as they were much shorter than the controls (data not shown). At E15.5, the mutant GTs were smaller in size, deformed (Figure 2D) and lacked the characteristic mesenchymal patterning present in controls (Figure S1A–S1B). To further explore the cellular basis for the reduction in GT size, we analyzed proliferation and cell-death in the GT of these mutants. We performed phospho-histone H3 (PHH3) staining on E11.0 coronal GT sections and counted the number of PHH3-positive cells in a fixed area. A 28% reduction in PHH3-positive cell number in the genital mesenchyme of the dcKOs was observed (Figure S1C–S1E, n=10, p = 0.0017). We also performed TUNEL analyses but did not observe any differences in the number of apoptotic cells between control and dcKO mutant GTs (data not shown).

An examination of genes known to mediate genital tubercle initiation revealed alterations in normal gene expression patterns as early as E11.5. Bmp4, Wnt5a and Msx1 were expressed in the PCM in control GTs (Figure 2E and 2G, and Figure S1F), and their expression was barely detectable in dcKO GTs (Figure 2F and 2H, and Figure S1G). Mxs2 was expressed in both the PCM and UE in controls (Figure S1H). In the GT-Fgfr1 IIc-2-dcKOs, Mxs2 expression was absent in the PCM and downregulated in the UE (Figure S1I). Moreover, UE expression of Shh was also downregulated in the dcKOs (Figure 2I–2H). In contrast, dUE-Fgfl8 expression remained unchanged in the GT-Fgfr1 IIc-2-dcKOs (Figure 2K–2L), suggesting that maintenance of Fgfl8 expression is independent of FGFR1 and FGFR2 during GT development. Collectively, these data demonstrate that FGF signaling is obligatory for promoting proximodistal GT outgrowth and for maintaining genital-specific gene expression.

**FGF Targets Genital Mesenchyme during GT Outgrowth**

The Msox2TakAPeO-Cre system mediates gene deletion in both the cloacal endoderm as well as the PCM. Therefore, it is not clear whether changes observed in the aforementioned Fgfr1 IIc-2-dcKOs were direct results of compromised FGF responsiveness in the PCM, or secondary to loss of FGF receptors in the cloacal endoderm. Thus, we tested the requirement for FGF responsiveness in these two compartments by using a previously characterized endoderm-specific Shh EGFPCre allele [4,33], and a PCM-specific Dermo1Cre allele [4,32], respectively. Shh EGFPCre/Fgfr1 IIc/Fgfr2 IIc (UE-Fgfr1 IIc-2-dcKO) and Dermo1Cre/Fgfr1 IIc/Fgfr2 IIc (PCM-Fgfr1 IIc-2-dcKO) mutants were generated and their GTs analyzed. Intriguingly, GTs from UE-Fgfr1 IIc-2-dcKOs did not exhibit any morphological abnormalities in the early outgrowth phase, and their size and shape were comparable to stage-matched controls (Figure S2A–S2D). Histological analysis revealed normal patterning of the genital mesenchyme in UE-Fgfr1 IIc-2-dcKOs embryos (Figure S2E and S2F). The only phenotype observed in these mutants was the abnormal maturation of urethral epithelium similar to what was observed in Fgf2IIb mutants [28], which will be discussed in a separate manuscript. Furthermore, regulatory genes including Msox2, Bmp4, Wnt5a and Fgfl8, were also properly expressed in these UE-Fgfr1 IIc-2-dcKO embryos (Figure S2G–S2J).

In contrast, the GTs of PCM-Fgfr1 IIc-2-dcKO were clearly smaller than their littermate controls (Figure S3A–S3B). Further analyses revealed that the distal GT mesenchymal expression of P-ERK1/2, a previously established FGF target gene [26], was also downregulated in these mutants (Figure S3C–S3D). In addition, PHH3 staining on E11.5 embryos revealed a 20% reduction in mitotic figure number in the PCM-Fgfr1 IIc-2-dcKOs (Figure S3E–S3G). Collectively, these data indicate that the main target for FGF signaling during GT outgrowth is the PCM. A similar requirement for FGF signaling in the limb mesenchyme has been described previously [34].

![Figure 2](image-url)  
**Figure 2. Defective GT development in GT-Fgfr1 IIc-2-dcKO embryos.** (A–F) SEM analyses on age-matched control (A, C) and dcKO mutants (B, D) showing a retarded proximodistal outgrowth in the mutants. (E–L) Whole mount in situ hybridization on E11.5 control and dcKO mutants using probes indicated. Note the downregulation of Bmp4 and Wnt5a in the PCM (F and H), and Shh in the UE of the dcKO mutants (J). The dUE-Fgfl8 expression in the dcKO (L) was comparable to the control (K). Bars represent 500 μm.

doi:10.1371/journal.pgen.1003231.g002
FGF8 Is Sufficient to Regulate GT Development

Fgf8 is normally expressed in the distal-posterior cloacal endoderm at E10.5, and then in the dUE through E11.5–E14.5. We sought to determine whether the PCM could respond to dUE-expressed Fgf8 in vivo. We generated a conditional Fgf8 over-expressor mouse line by knocking the Fgf8 full-length cDNA (Accession: BC048734) into the ubiquitously expressed Rosa26 locus, preceded by a floxed transcriptional stop cassette (R26flKo). This design allows ectopic Fgf8 expression upon Cre-mediated recombination (Figure 3A). We used a tamoxifen (Tm)-inducible and 3E) was the result of ectopic expression in the anterior cloacal endoderm (arrow in Figure 3C evidenced by whole-mount in situ hybridization. The ectopic Fgf8 expression domain of this ShhCreERT2 allele [3,4,33] and generated and gene expression analyses. Hematoxylin and Eosin (H&E) staining was in sharp contrast to our previous observation in the UE-R26flKo-GOF mutants where ectopic up-regulation Fgf8 was sustained in the UE-R26flKo-GOF embryos showing excessive limb development in the mutants. The postaxial extra digit in the forelimb is indicated by an asterisk in J, and the ectopic skeletal components were indicated by arrows in J and K. The arrowhead in K indicates an enlarged calcaneus. h, humerus; r, radius; u, ulna; ft, femur; t, tibia; f, fibula. Bars represent 500 μm.

doi:10.1371/journal.pgen.1003231.g003

Figure 3. Appendage over-development in the R26flKo-GOF mutants. (A) A schematic diagram for R26flKo allele. (B–G) Whole mount in situ analyses on control (B, D, and F) and UE-R26flKo (C, E, and G) embryos at consecutive time points after Tm treatment. Note the up-regulation of PCM-Bmp4 (inset in C) and dUE-Fgf8 (C, arrowhead) in the UE-R26flKo embryos 8 hours after Tm administration, and a down-regulation of dUE-Fgf8 expression in the UE-R26flKo mutants 16 (E, arrowhead) and 24 hours (G) after Tm treatment. The ectopic Fgf8 expression in the anterior cloacal endoderm is indicated by arrows in C and E. (H–I) SEM on E14.5 control (H) and UE-R26flKo (I) embryos. Note that the GT in the GOF mutant is bigger. (J–K) Skeleton staining of E18.5 AER-R26flKo-GOF embryos showing excessive limb development in the mutants. The postaxial extra digit in the forelimb is indicated by an asterisk in J, and the ectopic skeleton components were indicated by arrows in J and K. The arrowhead in K indicates an enlarged calcaneus.
stained E12.0 transverse GT sections showed that the morphology of the rescued GT closely resembled that of the controls with the urethra properly positioned at the ventral side of the GT (Figure 4D). In addition, Hoxa13 and Hoxd13, both markers of the GT, were expressed in the rescued genital tubercles (Figure 4E and 4F). Altogether, these data indicate that restoring FGF8 rescued GT agenesis caused by β-catenin deficiency. It is noteworthy that this rescue of β-Cat-KO by FGF8 is confined to the GT, as other caudal malformations observed in the β-Cat-KO including failed cloaca seption and defective tail formation, were still present in the UE-β-Cat-LOF;R26Fgfr8 embryos (data not shown).

To test whether forced Fgf8 expression could also rescue limb deficiency caused by β-Cat ablation, we generated Mx2-Cre; β-Cat+/+ (AER-β-Cat-LOF) and Mx2-Cre; β-Cat+/+;R26Fgfr8+ (AER-β-Cat-LOF;R26Fgfr8+), both markers of UE-R26Fgfr8-LOF) mutants. Consistent with a previous investigation [3], all autopod elements, radius, and distal two-thirds of the ulna were missing from the forelimbs of AER-β-Cat-LOFs (Figure 4I), whereas the humerus was thinner and lacked the deltoid tuberosity (Figure 4I compared to G, arrow). In contrast, the LOF embryos with the R26Fgfr8 allele developed normal humeri with the deltoid tuberosity (Figure 4K, arrow). The radius was evident and the ulna was longer and thicker (Figure 4K). Moreover, several small pieces of alcian blue-stained cartilage were observed distal to the ulna, indicating the presence of autopod rudiments (arrowhead and inset in Figure 4K). The hindlimbs of the AER-β-Cat-LOFs were largely absent except for a small remnant of the pelvic girdle (Figure 4I), whereas the R26Fgfr8-expressing β-Cat-LOF embryos developed near normal pelvic girdles and femurs (Figure 4I) along with one or two ectopic cartilages (asterisk in Figure 4I). These phenotypes were consistently observed in all AER-β-Cat-LOF;R26Fgfr8 embryos (n = 10), and indicated that exogenously supplying FGF8 can partially restore distal limb structures lost in the AER-β-Cat-LOF embryos. Collectively, these data suggest that FGF8 can promote outgrowth of both the GT and the limb in the absence of canonical epithelial WNT activity, suggesting that it functions as a downstream effector of WNT signaling during limb and GT outgrowth.

**Growth-Promoting Role for SHH Is Independent of FGF during Appendage Outgrowth**

The positive feedback loop involving FGFs and SHH plays a critical role in appendage outgrowth, as evidenced by the down-regulation in Fgf expression in both the AER [21,22,35,37] and the DU [5,6] of Shh-KOs, which display reductions in both appendages. We next examined whether forced Fgf8 expression could also rescue the severe appendage deficiencies caused by the absence of SHH. We generated ShhCreERT2/+;R26Fgfr8+ (Shh-KO;UE-R26Fgfr8) embryos, which allowed us to induce Fgf8 expression in the UE of Shh null mutants (ShhCreERT2 allele is also a null allele). However, we detected neither tubercle formation (Figure S3B), nor Hoxa13 or Hoxd13 expression (Figure S5C and S5D) in the cloacal region of these compound mutants at E12.5, 48 hours after Tm treatment. We also generated Mx2-Cre; R26Fgfr8+/+; ShhCreERT2/+;R26Fgfr8+ (Shh-KO;AER-R26Fgfr8) mutants, to test whether sustaining Fgf8 expression in the AER of Shh mutants can restore limb development. We carefully analyzed four Shh-KO embryos carrying both Mx2-Cre and R26Fgfr8 alleles, and found no evidence of more advanced limb development (Figure S5G and S5H), compared to thirteen Shh-KO mutants without R26Fgfr8 alleles (Figure S5E and S5F). Together, these results indicated that although Shh and Fgf8 expression are interdependent during appendage outgrowth, their function in promoting appendage outgrowth is independent and non-redundant.

**GT-Specific Gene Expression Depends on dUE-FGF Signals**

We next analyzed GT gene expression in UE-β-Cat-LOF; R26Fgfr8+ mutants to further interrogate the genetic networks underlying GT development and identify downstream targets of dUE signaling. Fgf8 expression was detected in the distal cloacal endoderm in controls (Figure 5A), but was absent in UE-β-Cat-LOF mutant (Figure 5B) at E10.5. The R26Fgfr8-expressing LOF embryos, on the other hand, showed very weak Fgf8 expression throughout the cloacal endoderm (arrow in Figure 5C). This low-expression was consistent with what we observed in the
UE- R26\(^{Fgf8}\)-GOF embryos, suggesting that these Fgf8 transcripts were transcribed from the R26 locus. Expression of Bmp4 and Wnt5a was normally detected in the PCM (Figure 5D and Figure S6A), absent in the UE-β-Cat-LOFs (Figure 5E and Figure S6B), and partially restored by ectopically supplying Fgf8 expression from the R26\(^{Fgf8}\) allele (Figure 5F and Figure S6C). The Shh pathway was also compromised in the β-Cat-LOF mutants. Shh (Figure 5H) and Ptc1 expressions (Figure S6E) were markedly decreased in the cloacal endoderm and the PCM, respectively. On the other hand, in the LOF embryos with the R26\(^{Fgf8}\) allele, the expression of Shh was partially restored in the UE (Figure 5I) and the PCM expressed Ptc1 restored to a level comparable to controls (Figure 5F).

Combined, these data indicate that most genes differentially regulated in the UE-β-Cat-LOF mutants were responsive to FGF8 induction, suggesting that their expression is controlled by the dUE-FGF signals. However, we found that Sp8, a transcription factor normally expressed in the cloacal endoderm (Figure 5J), was lost in the UE of UE-β-Cat-LOF mutant (Figure 5I), and did not respond to FGF8 supplementation (Figure 5L).

**SP8 Is Required for Appendage Outgrowth**

Sp8 is expressed throughout the cloacal endoderm and later in the UE (Figure 6A), overlapping with the Fgf8-expressing dUE during GT development. Previous studies have implicated Sp8 in the transcriptional regulation of Fgf8 expression in the mouse commissural plate [39] and the chick limb ectoderm [39]. These findings prompted us to explore its role in mediating the WNT-β-Cat pathway. We first examined Sp8 expression in the UE-β-Cat-LOF and GOF-mutants (for GOF analyses, we used a previously established β-Cat-lox allele which produces stabilized β-catenin upon Cre-mediated recombination [40]). We found that Sp8 expression was reduced in the β-Cat-LOF UE (Figure 6B) and increased in the β-Cat-lox\(^{β-Cat}\)-GOF UE (Figure 6C). Similarly, robust Sp8 expression was also detected in the AER (Figure 6D), markedly reduced in the AER-β-Cat-LOF mutants (Figure 6E), and upregulated in the β-Cat-lox\(^{β-Cat}\)-GOF mutants where β-catenin activity was ectopically augmented in the limb ectoderm (Figure 6F, inset showing ventral view of a hindlimb). These data indicated that Sp8 is downstream of Wnt-β-catenin signaling in the UE and the limb ectoderm.

Next, we examined the GT phenotype of Sp8-null (KO) mutants. We found that 14/36 mutant embryos examined between E12.5–E15.5 exhibited GT agenesis (Figure 6H), while the rest demonstrated a range of GT defects including deformation, hypoplasia and proximal hypospadias (data not shown). Fgf8 expression was completely absent in the cloacal endoderm in all Sp8 KO embryos examined at E11.5 (Figure 6J, n = 9). Notably, these embryos also exhibited other caudal malformations such as deformed perineum, anal channel and tails, raising the concern whether the observed GT defects were secondary to earlier cloacal or neural tube malformations. Thus, we employed a conditional Sp8 null allele with the Shh\(^{CreERT2}\) line to generate UE-β-Cat-LOF mutants, and induced Sp8 deletion by tam treatment around the time of GT initiation (E10.5). GTs from the UE-β-Cat-LOF mutants were smaller than their age-matched controls, especially at the distal tip (Figure 6L). We also found a clear reduction in Fgf8 expression in these mutants by in situ analyses (inset in Figure 6L).

Consistently, the expression domains of Wnt5a (Figure S7B) and Msx2 (Figure S7D), both maintained by FGF8 from the neighboring dUE, were reduced.

Limb truncation, attributed to a failure to form the AER and consequently loss of Fgf8 expression, has been previously described in Sp8\(^{-/-}\) embryos [41]. We generated AER-Sp8-LOFs using the
Msx2-Cre line, and these mutants also showed a defect in limb outgrowth as evidenced by a loss of distal structures (Figure 6Q–6S). The stylopod and zeugopod developed normally in the forelimbs, while typically only one abnormal digit formed in these mutants (Figure 6Q–6R). The tibia and fibula were either missing or severely truncated, and no autopod was observed in the hindlimbs (Figure 6S). Notably, these limb defects can also be partially rescued by over expression of Fgf8. Skeleton preparation of E18.5 embryos showed that the AER-Sp8-LOF embryos carrying R26Fgf8 allele developed three digits in the forelimb (Q and R), and all zeugopod and autopod elements in the hindlimb (S); whereas in the R26Fgf8-rescued LOF mutants, several digits were formed in the forelimb (T, U), and the tibia and fibula were fully developed along with some autopod rudiments in the hindlimb (V, W). h, humerus; r, radius; u, ulna; fm, femur; t, tibia; f, fibula. Bars represent 500 μm in G and H, 400 μm in K and L.

doi:10.1371/journal.pgen.1003231.g006

Figure 6. Appendage deficiencies in Sp8 mutants. (A–F) Whole mount Sp8 in situ showing a downregulation of Sp8 expression in the UE and the AER of corresponding β-Cat-LOF mutants (B, E), and an upregulation of Sp8 expression in the UE (C) and the ventral limb ectoderm (F) of β-Cat-GOF mutants. Note the tail expression of Sp8 remains unchanged in (E compared to D). (G, H) SEM on E15.5 embryos showing an absence of GT in the Sp8 KO mutant (H). (I, J) Fgf8 in situ showing loss of expression in E11.0 Sp8 KO mutant. (K, L) SEM on E13.5 embryos showing a mild distal reduction in the UE-Sp8-LOF (L). Insets showing whole mount Fgf8 in situ on E12.5 control (K) and UE-Sp8-LOF (L) GTs. Note the downregulation of Fgf8 in the UE-Sp8-LOF mutant (L). (M–W) Skeletal preparation of E18.5 embryos showing that AER-Sp8-LOF lacked most autopod elements in the forelimb (Q and R), and all zeugopod and autopod elements in the hindlimb (S); whereas in the R26Fgf8-rescued LOF mutants, several digits were formed in the forelimb (T, U), and the tibia and fibula were fully developed along with some autopod rudiments in the hindlimb (V, W). h, humerus; r, radius; u, ulna; fm, femur; t, tibia; f, fibula. Bars represent 500 μm in G and H, 400 μm in K and L.

doi:10.1371/journal.pgen.1003231.g006

Sp8 Mediates WNT-Induced Fgf8 Expression

To test whether Sp8 is in the same genetic pathway with β-catenin and Fgf8, we sought to determine whether Sp8 mediates WNT-induced Fgf8 expression in the dUE and AER. We generated compound mutants using the β-Catlox allele, which we have previously shown to activate Fgf8 expression in both the UE and the limb ectoderm, together with the floxed Sp8 allele and the corresponding Cre lines. We compared Fgf8 expression in embryos with one β-Catlox allele and different numbers of functional Sp8 alleles by real-time RT-PCR and in situ hybridization. We found that the level of WNT-induced Fgf8 expression in the UE positively correlated with the number of functioning Sp8 alleles (Figure 7A–7E). Removing one wild type Sp8 allele caused a twofold reduction in Fgf8 expression, whereas ablating both wild type Sp8 alleles reduced Fgf8 expression by more than three-fold (Figure 7E). These results were further verified by Fgf8 in situ hybridization (Figure 7A–7D).

Similarly, deleting both Sp8 alleles abolished the ectopic Fgf8 expansion in the limb ectoderm of Msx2-Cre; β-Catlox/lox (AER-β-Cat-GOF) embryos (Figure 7F–7H), and consequently attenuated the polydactyly phenotype caused by constitutively active canonical WNT signaling (Figure 7I–7O). With two wild type Sp8
alleles, AER-\(\beta\)-Cat-GOF mutants developed an average of 6.8 digits in the forelimbs, and 6.1 digits in the hindlimbs (Figure 7J and 7M). In thirty percent of the embryos, ectopic limb in the flank ectoderm and ventral ectoderm were detected (Figure S8A and S8B). In comparison, AER-\(\beta\)-Cat-GOF;Sp8-null mutants only developed 4.9 digits in the forelimbs and 4.2 digits in the hindlimbs (Figure 7K and 7N, 28% and 31% reduction compared to AER-\(\beta\)-Cat-GOFs, respectively). In addition, no extra limbs were observed at ectopic locations. Collectively, these results indicate that SP8 is responsible, at least in part, for the WNT-induced activation of Fgf8 expression during appendage outgrowth.

To test whether augmented Sp8 expression alone can induce Fgf8 overexpression, we generated a R26Sp8 allele to conditionally overexpress Sp8 using the same strategy as for the R26Fgf8 line (Figure 8A). Mice carrying ShhEGFPCre or Msx2-Cre alleles were used to generate corresponding UE-R26Sp8-GOF and AER-R26Sp8-GOF embryos. Overexpression of Sp8 in the UE and AER was confirmed by in situ hybridization (Figure 8C, and data not shown). However, unlike Fgf8- or \(\beta\)-Cat-GOF embryos, Sp8-GOF embryos showed normal development in both appendages (Figure 8E and 8I). Fgf8 expression in the dUE and the AER of the corresponding Sp8-GOF mutant was also comparable to their wild type counterparts (Figure S9A–S9D).

We also crossed the R26Sp8 allele into the UE- or AER-\(\beta\)-Cat-LOF mutants to test whether forced Sp8 expression can bypass epithelial \(\beta\)-catenin to induce Fgf8 expression and initiate/maintain appendage outgrowth. We carefully analyzed six compound mutants and did not observe phenotypic rescue in either the GT (Figure 8F) or the limb (Figure 8J–8M). In addition, no Fgf8 induction was detected in the dUE of the corresponding \(\beta\)-Cat-LOF mutants carrying R26Sp8 allele (Figure 8G). All of these results indicate that SP8 by itself is insufficient to activate Fgf8 expression, and suggest that SP8 is a facilitator of WNT-mediated Fgf8 activation during appendage formation.

**Discussion**

In this study, we investigated the molecular cascade that regulates distal signaling centers in appendage development. Our study elicited a conservative genetic circuitry involving WNT-\(\beta\)-catenin signaling, the transcription factor Sp8, and the growth factor Fgf8, that underpins proximodistal outgrowth of limbs and external genitalia.

**FGF8 Mediates FGF Signaling in GT Outgrowth**

Our work provides in vivo evidence that FGF signaling is indispensable for early GT outgrowth. These findings are consistent with results from organ culture studies that inhibition of FGF signaling caused an arrest in GT development [24]. Our data demonstrates that the PCM's ability to respond to an FGF signal is essential for normal early genital tubercle outgrowth, as removing FGF receptors from the PCM caused impaired cell proliferation and perturbed normal gene expression patterns which led to severe GT reduction.

The obligatory role for FGF signaling during tubercle morphogenesis underscores the importance of identifying FGF ligands
A Conserved Mechanism Regulating Appendage Outgrowth

We have demonstrated a conserved WNT-SP8-FGF8 pathway in the distal signaling epithelia that functions to promote proximodistal outgrowth of both limbs and genitalia. We and others have shown that the canonical WNT pathway is a master molecular switch in the signaling epithelia during appendage formation, as epithelial WNT activation is not only necessary but also sufficient to induce Sp8 and Fgf8 expression and appendage outgrowth. FGF8 is the downstream signal output for the WNT pathway during this process as it acts directly on recipient mesenchymal cells to promote cell proliferation and establish patterns of gene expression. This genetic hierarchy has been supported by our observation that forced overexpression of Fgf8, even at a level much lower than endogenous expression, can bypass the requirement for epithelial WNT-β-catenin signaling to activate gene expression and initiate/maintain appendage outgrowth. Notably, in both appendages, the rescue of β-catenin deficiency by ectopic Fgf8 expression is only partial. This could be due to either weak Fgf8 expression from the R26 locus compared to the endogenous level of expression, or the possibility that in addition to regulating Fgf8 expression, canonical WNT signaling is also required for the formation of the AER structure, independent of Fgf signaling [20]. The regulation of Fgf8 by the canonical WNT-β-catenin signaling pathway is in part mediated by SP8, as Sp8 expression is regulated by WNT signaling in both limb

Figure 8. Construction and characterization of R26Sp8 knock-in allele. (A) A schematic diagram for R26Sp8 allele. (B, C) Sp8 in situ on E12.5 GTs showing an upregulation of Sp8 expression in the UE-R26Sp8 embryos (C). (D–F) SEM analysis on E15.5 embryos showing comparable GT development between control and the UE-R26Sp8 mutant (D), and no tube formation in the UE-β-Cat-LOF mutant carrying R26Sp8 allele (E). (G) Fgf8 in situ showing no Fgf8 expression in the UE-β-Cat-LOF/R26Sp8 mutant. (H–M) Skeletal preparation showing no difference between control (H) and AER-R26Sp8 mutant (I), or between the AER-β-Cat-LOF mutants with (K, M) or without the R26Sp8 allele (J, L). Bars represent 500 μm in D and E, 400 μm in F.

doi:10.1371/journal.pgen.1003231.g008

ing all the available evidence, we conclude that FGF8 produced by the dUE is most likely the endogenous ligand that mediates FGF responses during GT development.

Notably, the GT phenotype of the UE-Fgfr1;2-ΔcKO embryos is less severe than what was observed previously in Fgf4;Fgf8-Fgfr2IIIb-cKOs [23]. This difference is likely attributable to the method of gene ablation since in the Fgfr2IIIb-cKO embryos, Fgfr2-IIIb is abolished from not only the UE, but also from the ventral ectoderm. The underdeveloped phenotype described in Fgf4;Fgf8-Fgfr2IIIb-cKO embryos reflects a deficiency from ectodermal FGF responsiveness as it can be phenocopied by conditional ablation of both Fgf8 and Fgf2 using the ectodermally restricted Mox2-Cre allele (Lin et al., unpublished data).

Implication of a Conserved WNT-SP8-FGF8 Pathway in Appendage Outgrowth

We have demonstrated a conserved WNT-SP8-FGF8 pathway in the distal signaling epithelia that functions to promote proximodistal outgrowth of both limbs and genitalia. We and others have shown that the canonical WNT pathway is a master molecular switch in the signaling epithelia during appendage formation, as epithelial WNT activation is not only necessary but also sufficient to induce Sp8 and Fgf8 expression and appendage outgrowth. FGF8 is the downstream signal output for the WNT pathway during this process as it acts directly on recipient mesenchymal cells to promote cell proliferation and establish patterns of gene expression. This genetic hierarchy has been supported by our observation that forced overexpression of Fgf8, even at a level much lower than endogenous expression, can bypass the requirement for epithelial WNT-β-catenin signaling to activate gene expression and initiate/maintain appendage outgrowth. Notably, in both appendages, the rescue of β-catenin deficiency by ectopic Fgf8 expression is only partial. This could be due to either weak Fgf8 expression from the R26 locus compared to the endogenous level of expression, or the possibility that in addition to regulating Fgf8 expression, canonical WNT signaling is also required for the formation of the AER structure, independent of Fgf signaling [20]. The regulation of Fgf8 by the canonical WNT-β-catenin signaling pathway is in part mediated by SP8, as Sp8 expression is regulated by WNT signaling in both limb

important for GT development. Our data are consistent with the views of Haraguchi et al. [24], suggesting that dUE-expressed Fgf8 plays a key role in promoting GT outgrowth. Fgf8 is expressed at the correct time and place to signal to the PCM, which expresses both Fgfr1 and Fgf2. This is in conflict with recent data by Seifert et al. [25] in which they suggested that a normal GT could develop in the absence of Fgf8 expression. They did not detect FGF8 protein, which led to the conclusion that the Fgf8 mRNA may be present but not actively translated by the UE. They proposed that the ventral ectoderm may be an alternative source of FGF8 during normal development. The inability to detect FGF8 in the dUE by IHC is likely caused by the low expression level of Fgf8. We found that in E10.5 mouse embryos, robust AER Fgf8-expression was observed 2 hours after incubation with Alkaline Phosphatase substrates following standard in situ hybridization procedures, whereas dUE-expression was not observed until 12–18 hours later. In addition, the extensive genetic redundancy among FGFs has to be carefully considered. A recent study demonstrated ectopic Fgf4 expression in the dUE of Fgf8-cKO embryos, and ectopic Fgf8 expression in the dUE of Fgf4;Fgf8-ΔcKO embryos [26]. Both FGF4 and FGF3 can efficiently induce mitogenic activity when paired with FGFR1 or FGFR2 in vitro [42]. These observations indicate that not only FGFs endogenous to the dUE can compensate for the loss of Fgf8, other FGFs can also be ectopically activated to fulfill the requirement for FGF signaling. Activities from these ectopic FGFs could well explain the lack of GT phenotype in Fgf8-cKO mutants. Intriguingly, induction of Fgf3 and Fgf4 was not observed in either β-Cat- or Sp8-cKOs (Figure S10A–S10D), while the dUE expression of Fgf9 was downregulated in Sp8-cKOs (Figure S10E–S10F). These results suggest that the upregulation of these compensatory FGF factors also requires WNT and SP8. Alternatively, the hypothesis that FGFs can be produced by the ventral ectoderm is plausible [25]. However, one has to keep in mind that the GT is built and patterned around the UE. Most regulatory genes expressed distally including Msx1, Msx2, Wnt5a and Lf1, showed peri-dUE expression but not sub-ectodermal expression. Consistently, we have shown that the expression of these genes is orchestrated by UE-specific WNT and FGF signaling. Considering all the available evidence, we conclude that FGF8 produced by the dUE is most likely the endogenous ligand that mediates FGF responses during GT development.
ectoderm and the UE, and is necessary for Fgf8 expression and subsequent appendage formation. However, unlike WNT activity and Fgf8 expression, the expression domain of Sp8 is not restricted to the AER and dUE but also includes the limb ectoderm [41] as well as proximal UE (Figure 6A), suggesting that Sp8 is a permissive but not inductive factor for the establishment of Fgf8 expression. In support of this notion, overexpressing Sp8 in the limb ectoderm or UE did not cause any perturbation in Fgf8 expression or appendage formation. In addition, forced Sp8 expression failed to rescue Fgf8 expression and appendage outgrowth in the epithelial β-Cat-LOF mutants. Notably, even with both Sp8 alleles mutated, the dUE and AER Fgf8 expression in the β-Cat-FOX mutants is still higher than in the controls. This finding apparently is counterintuitive to the obligate role for Sp8 in maintaining Fgf8 expression during normal outgrowth processes. One possible explanation is that the level of WNT signaling induced by the stabilized β-catenin protein from the β-Catβex3 allele might be too high, and not subjected to endogenous regulatory mechanisms. This could potentially trigger ectopic events that lead to Fgf8 overexpression. It is also noteworthy that the other members of SP/KL transcription factor family Sp6, was upregulated in the β-Cat-GOF mutants (Figure S11). Sp6 is expressed in the cloacal endoderm and the AER in early appendage development (Figure S11B and Figure S6A, respectively), and loss of Sp6 causes abnormal AER formation and Fgf8 expression [43]. Similar to Sp8, the expression of Sp6 is also downstream of the canonical WNT signaling pathway but independent of FGFs in the AER [43]. The exact function of Sp6 in regulating GT development and dUE-Fgf8 expression remains to be determined. However, it is plausible that high levels of Sp6 induced by ectopic WNT signaling might compensate for the absence of Sp8 in the regulation of Fgf8 expression.

The exact molecular mechanisms through which Fgf8 expression is regulated by Sp8 and β-catenin, and how Sp8 expression is regulated by WNT-β-catenin remain to be determined. Direct binding of the β-catenin/LEF1 complex to cis-regulatory elements within the Fgf8 promoter has been reported in dental epithelial cell lines [44] and nephron progenitors [45], suggesting that this WNT-Fgf8 pathway also functions in the development of other organs involving epithelial-mesenchymal interactions. In our preliminary studies, we identified several novel LEF binding sites around Fgf8 and Sp8 genes (data not shown). The functional relevance of these binding sites in GT and limb development will be the goal of future investigations.

Although the distal signaling cascades appear to be similar in both appendages, the upstream events leading to their initiation are likely different. In the limb, the ectoderm is the site for both Wnt3 expression [3,46], and the induction of canonical Wnt downstream targets as evidenced by TOPGAL activity [47]. On the other hand, the establishment of the dUE signaling center in the cloacal endoderm appears to involve signal transduction between the cloacal endoderm and the ventral ectoderm. Both TOPGAL and Fgf8 expression are confined to the endodermal cells adjacent to the genital ectoderm [4,24], and the contact between ectoderm and endoderm appears to be a prerequisite for Fgf8 induction and GT initiation [25]. In support of this notion, candidate WNT ligands responsible for activating canonical WNT signaling in the dUE, Wnt3 and Wnt7a, are both expressed in the genital ectoderm [4,26].

Altogether, our results demonstrate extensive parallels between genetic networks regulating the outgrowth of both the limb and GT. These findings strongly support the notion initially brought up by two pivotal studies on the role of Hox genes in appendage development [10,11], that mammalian GT development appears to be achieved through co-option of the limb outgrowth program, i.e. the mammalian GT and limb share deep homology [48,49].

Materials and Methods

Construction of R26CreSp8 and R26Sp8 Alleles

Clones containing full length Fgf8 (BC048734) and Sp8 (BC082582) cDNAs were obtained from Invitrogen (Carlsbad, CA). The cDNAs were released and subcloned into the NotI site of the pBIGT vector [50]. The insert containing either Fgf8 or Sp8 cDNA and a floxed transcription stop cassette was released by Pad1/Ase1 double digestion and cloned into pRosa26-PA [51]. The targeting construct was linearized by Swal and subjected to electroporation (performed by ES cell core at Washington University). The ES cells were screened for recombination by PCR and southern blotting. Five out of seventy-two clones were positive for recombination for R26Fgf8 allele, and six out of sixty clones were positive for R26Sp8 allele. Positive clones were expanded, karyotyped and used for blastocyst injection. For both lines, at least three chimeras were able to pass the knock-in alleles through germline transmission, and the phenotypes resulting from expression of the knock-in alleles were identical. For all experiments described in the manuscript, three embryos with the same genotype were examined if not otherwise specified.

Animal Maintenance and Treatments

All animals were maintained according to NIH guidelines and in compliance with animal protocol approved by Washington University. Mx2rtTA [29], Sp6+/− and Sp6−/− [41], teto-Cre [52], β-Cat+/- [53], β-CatEx3/Wnt1 Ex3 [40], ShhCreERT2 and ShhEGFPCre [33], Mx2-Cre [35], Fgf10f/f [31], Dermo1Cre and Fgf2f/f [32] alleles were previously described. Tamoxifen treatment and doxycycline treatment were performed as previously described [30].

Real-time RT-PCR

For all experiments, we used three independent biological samples from each genotype. Each sample contained a pool of RNA isolated from two E12.5 GTs with the corresponding genotype. The results were analyzed using the delta-CT method. Expression of the corresponding genes was normalized to that of housekeeping gene Rpl7.

Whole-Mount In Situ Hybridization

Whole mount in situ hybridization was performed using a standard protocol. The probes were previously described [4].

Anatomical and Histological Analyses

Paraformaldehyde (4%)-fixed embryos were dehydrated and embedded in paraffin. Five-micron transverse GT sections were embedded in paraffin. Five-micron transverse GT sections were analyzed using a standard microscope. Scanning electron microscopy analyses were performed as previously described [4].

Skeleton Preparation

Skeleton preparation was performed as previously described [54].

Accession Numbers

The Genbank accession numbers for genes included in the manuscript are as follows: Fgf8 (NM_010205.2), Sp8 (NM_177082.4), β-catenin (NM_007614.3), Fgf1 (NM_010206.2), Fgf2 (NM_010207.2), Shh (NM_009170.3).
Supporting Information

Figure S1 Defective mesenchymal patterning and proliferation in GT-Fgfr1;2-dcKO embryos. (A–B) E15.5 histological analyses showing patterned mesenchymal condensation in control GT (E), but not the dcKO GT (B). (C–E) PHH3 staining of E11.0 control and dcKO GTs showing a 28% reduction in the number of PHH3-positive mesenchymal cells in a fixed region (n=10, p = 0.0017). (F–I) Whole mount in situ on E11.5 control and dcKOs showing downregulation of the P-ERK1/2 in the distal GT mesenchyme in the mutants. (A–C) PHH3 staining on E11.0 control and UE-Fgfr1;2-dcKO mutants (p = 0.017). (D–I) Histological analyses on E15.5 GT transverse sections showing no difference in size or patterning of the GT mesenchyme between the control (E) and mutant (F). (G–J) Whole mount in situ hybridization on E11.5 UE-Fgfr1;2-dcKOs revealing normal expression of regulatory genes. (JPG)

Figure S2 Phenotype of UE-Fgfr1;2-dcKOs. (A–D) SEM analyses revealed no gross morphological differences between controls and the dcKO mutants at E13.5 (A, B), and E15.5 (C, D). (E, F) Histological analyses on E15.5 GT transverse sections showing no difference in size or patterning of the GT mesenchyme between the control (E) and mutant (F). (G–J) Whole mount in situ hybridization on E11.5 UE-Fgfr1;2-dcKO revealing normal expression of regulatory genes. (JPG)

Figure S3 Defective GT outgrowth in the PCM-specific Dermo1-Cre; Fgfr1;2-dcKOs. (A–B) SEM on E14.5 control and PCM-Fgfr1;2-dcKO showing an underdeveloped GT in the mutant (B). Note that the P-D outgrowth was deficient in the mutant. (C–D) Phospho-ERK1/2 staining on E11.5 coronal GT sections showing downregulation of the P-ERK1/2 in the distal GT mesenchyme in the mutant (D). (E–G) PHH3 staining showed a 20% reduction in mitotic figure number in the dcKO mutants (p = 0.017). (JPG)

Figure S4 Phenotype of AER- and UE-specific Fgfr3-GOF mutants. (A–C) PHH3 staining on E11.0 control and UE-R26β-Cat± embryos showing a 19% increase in number of PHH3-positive cells in the control GT (n=10, p = 0.009). (D–E) SEM analyses on E12.5 UE-R26β-Cat± and control GT showing an overdeveloped GT in the MUTANT. (F–G) Skeletal preparation of E18.5 control and AER-R26β-Cat± embryos showing ectopic bone (arrow) development. (JPG)

Figure S5 Phenotype of Shh-KO mutants with forced Fgfr3 expression. (A, B) SEM analyses showing no tubercle formation in Shh-KO mutants with (A) or without (B) R26β-CAT allele. (C, D) Hoxa13 (C) and Hoxd13 (D) in situ showing no expression in the Shh-KO bearing R26β-CAT allele. (E–H) Skeleton staining showing no difference in limb development between Shh-KO with (E, F) or without (G, H) AER-R26β-CAT expression. (JPG)

Figure S6 GT gene expression analyses on UE-β-CAT-LOF embryos with forced Fgfr3 expression. (A–F) Whole mount in situ using probes indicated. Note the absence of PCM Wnt5a (B) and Ptch1 (E) expression in the LOF mutants; and partially restored Wnt5a (C) and near normal Ptch1 (F) expression in the LOF mutants with R26β-CAT allele. (JPG)

Figure S7 GT gene expression analyses on UE-Sp8-LOF embryos. (A–D) Whole mount in situ on E12.5 embryos using probes indicated. Note the distal expression domains of both Msx2 (B) and Wnt5a (D) were both smaller in the mutant GTs. (JPG)

Figure S8 Excessive limb development in ectopic positions in the AER-β-CAT-GOF embryos. (A, B) Ectopic limb formation was indicated by arrows. (JPG)

Figure S9 Fgfr3 expression in R26β-CAT-GOF mutant. (A–D) Fgfr3 in situ on control (A, C), UE-R26β-CAT-GOF (B) and AER-R26β-CAT-GOF mutant (D) revealing comparable expression levels in the GT (A, B) and the forelimbs (C, D). (JPG)

Figure S10 Expression of Fgfs in UE-β-CAT- and Sp8-CAT-KOs. (A–F) In situ hybridization using probes indicated. Note no Fgfr (A–B) or Fgfr7 (C–D) expression was detected in either mutants (A positive control for the Fgfr7 in situ is shown in the inset of A), and downregulation of dUE Fgfr expression (arrow in E) in Sp8-CAT-KO (F). (JPG)

Figure S11 Sp6 expression in E11.0 WT embryos. (A, B) Whole mount Sp6 in situ showing AER expression (arrows in A) and UE expression (B). (JPG)

Acknowledgments

We thank Jaclyn Lett, and Washington University in St. Louis, Department of Otolaryngology, Research Center for Auditory and Visual Studies (funded by NIH P30 DC004665) for providing technical assistance on Electron Microscopy.

Author Contributions

Conceived and designed the experiments: CL, LM. Performed the experiments: CL, YY SMB GMV HC S-HH. Analyzed the data: CL, YY SMB LM. Contributed reagents/materials/analysis tools: SMB S-HH DMO LM. Wrote the paper: CL, YY SMB DMO LM.

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