In 1925, Charles Danforth wrote about “mice with six legs [that] appeared about two years ago in a stock which had descended from five individuals and had been inbred for several generations” [1]. Danforth studied this “duplicitas posterior” (including duplication of internal and external urogenital organs, with quadrilateral symmetry) and its genetic transmission for several years [2], but over time he derived a line in which the principal characteristic was dominant transmission of a short, kinky, or absent tail, etymologically described as the Danforth’s short tail (Sd) mutation [3] (Figure 1A and 1B). Internal caudal regression phenotypes are more serious and include defects in the skull tissue caused by early degeneration of the notochord; small, malformed or absent kidneys; and hindgut abnormalities. Homozygotes die shortly after birth with more severe phenotypes, including complete loss of the tail, loss of both kidneys, lack of innervation along sections of colon, imperforate anus, and persistence of an incisura ileocrotica—a developmentally transient structure in mammals important for urogenital development. Interestingly, analysis of chimeras indicated cell autonomy for defects in the spine and hindgut, but not in kidney [4].

Despite continued interest over several decades, Sd resisted efforts to identify the causal gene—until now. In this issue of PLOS Genetics, three laboratories independently identify the Sd mutation as an 8.5 kb ETn retrotransposon insertion 12.3 kb upstream from the Ptf1a gene [5–7]. Ptf1a encodes a cell type–restricted basic helix-loop-helix transcription factor required for development of the pancreas and cerebellum [8–10]. In the new work, all three groups conclude that ectopic expression of Ptf1a is the causal event in Sd mice.

Catherine Keegan and colleagues (Vlangos et al.) took advantage of the published Sd map location [11] (Figure 1C) and powerful genomic tools to isolate the mutation [5]. Finding no mutations after Sanger sequencing all the exons of annotated coding genes in the interval, they developed a custom oligonucleotide array to capture all non-repetitive sequences from the interval for massively parallel sequencing, using paired-end reads and requiring both ends to map correctly in the interval to ensure high-quality assembly (Figure 1D). This allowed the group to analyze more than half of the nucleotides in the critical region at a read depth of 440×, but still provided no plausible candidate mutations for Sd. Because de novo insertions of repetitive sequence are a frequent source of mutations in mice [12], the group reassembled paired-end samples that had been discarded in the first assembly pipeline because one end landed in a repeat. Using this new algorithm, they identified exactly one novel insertion, not present in a reference genome that shares extensive haplotype with the Sd chromosome, 12.3 kb proximal to Ptf1a. This candidate insertion was not found in any modern strains tested. RT-qPCR experiments showed a striking, dose-dependent increase in Ptf1a expression in Sd/+ and Sd/Sd embryos. Genomic and cDNA-based transgenics provide preliminary evidence that broad overexpression of Ptf1a causes embryonic lethality, possibly as an extreme example of the severe Sd/Sd phenotype.

Ali Gharavi and co-workers (Lugani et al.) took a complementary approach, performing SNP-based linkage mapping in 1,497 segregants to refine the Sd interval to a remarkably tidy 42.8 kb intergenic region [6] (Figure 1E). Complete Sanger sequencing of this interval by the group yielded a single DNA change relative to reference sequences: the 8.5 kb ETn element. The complete, high-confidence sequencing of the critical region provides a rigorous demonstration that the ETn insertion must be the Sd mutation. Both qPCR and in situ hybridization assays again confirmed strong ectopic expression of Ptf1a, encompassing all Sd-affected tissues. No other protein-coding genes in the broader region near the insertion site showed any similar change. A limited analysis of known Ptf1a transcriptional target genes failed to identify upregulation induced by the spread of Ptf1a in Sd embryos, but key Ptf1a targets in ectopic tissues need not be the same as those in its normal sites of expression.

Ken-ichi Yamamura’s group (Semba et al.) also began with a conventional positional cloning approach. By physical mapping with an Sd/Sd cosmid library constructed for that purpose, they found an unexpectedly large fragment containing the 8.5 kb ETn element as a candidate mutation [7]. To test its functional relevance to Sd, the group performed a true tour-de-force of mouse genetics, producing a series of targeted and transgenic alleles to determine which gene products were functionally important to the Sd phenotype in the context of the ETn (Figure 1F). As the first step of a serial targeting strategy [13], a neomycin resistance cassette flanked by mutant loxP sites was introduced at the same position as the ETn insertion. This did not induce an Sd-related phenotype, suggesting that simple disruption of a cis-acting sequence is unlikely to explain the defect. However,
Figure 1. The tale of Danforth’s short tail. (A) Mice from Danforth’s original stock showed posterior duplications, including duplicated hind limbs and a pelvic bulge, in addition to kinked and sometimes shortened tails. Drawing idealized from photographs in [2]. (B) Sd/+ mice show a strain-dependent range of caudal phenotypes, including kinked, shortened, or absent tail and reduction or loss of one kidney, but without pattern duplications. Sd/Sd animals die at birth with caudal regression, including malformation of vertebrae, absent tail, loss of both kidneys, persistent cloaca.
replacing the neo cassette with a fragment containing the Sd ETn produced an allele with dosage-sensitive short tail phenotypes, indicating that the insertion of the ETn at this location is sufficient to create an Sd-like mouse. However, in addition to Ptf1a, this group found overexpression in Sd embryos of two adjacent non-coding RNAs (ncRNAs), Gm13344 and Gm13336. A transgene including the ETn, Ptf1a, and Gm13336 (which overlaps Ptf1a on the opposite strand) was sufficient to induce caudal phenotypes, but a similar construct containing the ETn and the other ncRNA was not, narrowing the list of functional candidate genes to two. The team then created germine-competent ES cells from Sd/+ embryos and serially targeted the Ptf1a/Gm13336 overlap. Integration of a floxed neo cassette on the ETn haplotype creates a Ptf1a null with the expected pancreatic agenesis, but no tail or other Sd-like effects. However, replacing the neo cassette with Ptf1a, but not Gm13336, does phenocopy Sd, demonstrating that it is specifically the ETn-dependent expression of Ptf1a that triggers the developmental abnormalities that have been studied in Sd mice for more than 70 years.

Through what effectors does Ptf1a ectopic expression act? Sembia et al. provide an initial answer by profiling RNA in both Sd and ETn-Ptf1a transgenic mice relative to controls. They find down-regulation of Cdx2, another key transcription factor, along with three of its known activation targets, Cyp26a1, T, and Wnt5a. While it is not yet clear whether ectopic Ptf1a acts physically at Cdx2 in ectopic tissues, rather than indirectly through intervening factors, these profiling results provide clues to important pathways whose expression is disrupted as a consequence of Ptf1a-Sd. The results from all three groups, along with analyses of target pathways activated or repressed by Ptf1a in target tissues, will now allow us to ask how, or in which aspects, Sd accurately models human caudal malformation and regression syndromes.

Whether the ETn acts on Ptf1a by creating a broad enhancer or by blocking an endogenous silencing element remains to be determined. Either answer might provide insight into other ETn-induced regulatory mutations [14,15] or more broadly for integration of multiple cis-regulatory sites in the presence of retroelements. Additional serial targeting constructs that test activity of specific sequences in the ETn or perhaps chromatin condensation contact patterns (3G or its more sophisticated derivatives) might help to resolve the details here. In addition, most mutagenic ETn elements are much smaller [16,17] than that reported here, and the serial targeting strategy could be used to test whether more typical ETn elements confer a similar property to this locus. The discovery of the Sd mutation after so many decades might also prompt us to ask how often regulatory mutations might account for the remaining classical alleles that have been refractory to intragenic-centered analysis and exon sequencing.

The unusual nature of the Sd mutation also raises a final question: What relationship—if any—does Sd have to the original posterior duplication reported by Danforth, for which several specimens included completely duplicated hindlimbs, kidneys, gonads, phalli, and external urogenital openings? Did this stock contain multiple mutations, a more complex retrotransposon-mediated event that resolved into Sd, or other mutations unrelated to Sd? Perhaps that is another tail.

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