Analysis of the *vestigial tail* mutation demonstrates that *Wnt-3a* gene dosage regulates mouse axial development

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Mice homozygous for the recessive mutation *vestigial tail* (*vt*), which arose spontaneously on Chromosome 11, exhibit vertebral abnormalities, including loss of caudal vertebrae leading to shortening of the tail. *Wnt-3a*, a member of the *wingless* family of secreted glycoproteins, maps to the same chromosome. Embryos homozygous for a null mutation in *Wnt-3a* (*Wnt-3a^{neo}*) have a complete absence of tail bud development and are truncated rostral to the hindlimbs. Several lines of evidence reveal that *vt* is a hypomorphic allele of *Wnt-3a*. We show that *Wnt-3a* and *vt* cosegregate in a high-resolution backcross and fail to complement, suggesting that *Wnt-3a^{neo}* and *vt* are allelic. Embryos heterozygous for both alleles have a phenotype intermediate between that of *Wnt-3a^{neo}* and *vt* homozygotes, lacking a tail, but developing thoracic and a variable number of lumbar vertebrae. Although no gross alteration in the *Wnt-3a* gene was detected in *vt* mice and the *Wnt-3a* coding region was normal, *Wnt-3a* expression was markedly reduced in *vt/vt* embryos consistent with a regulatory mutation in *Wnt-3a*. Furthermore, the analysis of allelic combinations indicates that *Wnt-3a* is required throughout the period of tail bud development for caudal somitogenesis. Interestingly, increasing levels of *Wnt-3a* activity appear to be necessary for the formation of more posterior derivatives of the paraxial mesoderm.

[Key Words: Axial development; mouse; *vestigial tail* mutation; *Wnt-3a* gene; caudal somitogenesis; paraxial mesoderm]

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The vertebrae of the axial skeleton arise from segmented paraxial mesodermal precursors, the somites that are paired epithelial clusters lying on either side of the midline mesoderm of the notochord. Initially, the somite is an undifferentiated ball of cells. Subsequently, it undergoes a progressive regionalization generating dermal (dermatome), muscle (myotome), and cartilage (sclerotome) precursors in response to a complex network of signals. For example, the initiation of sclerotomal development, and consequently the formation of vertebrae, appears to be regulated by a notochord-derived signal—Sonic hedgehog (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995). Somite formation in the mouse is a protracted developmental process initiated at primitive streak stages in the early gastrulating embryo, 8.0 days post coitum (dpc), and continuing until 13.5 dpc (Tam and Tan 1992). When complete, ~65 somite pairs

are formed. These pairs give rise to a rostral–caudal progression of vertebrae generating an adult skeleton comprising 4 occipital, 7 cervical, 13 thoracic, 6 lumbar, 4 sacral, and ~30 caudal vertebrae that make up the tail (Gruneberg 1963; Rugh 1990).

Fate mapping studies indicate that the first somites formed are derived from mesodermal precursors arising at the anterior limit of the primitive streak (Tam and Beddington 1987; Tam 1989). As development continues, the primitive streak is gradually lost and by 9.0 dpc, or when the embryo has ~24–26 somites, a transition takes place wherein a new structure, the tail bud, contributes the mesodermal precursors that generate all of the remaining somites (Schoenwolf 1977; Tam 1984, 1986; Tam and Tan 1992). Thus, the occipital, cervical, and thoracic vertebrae are probably derived from the primitive streak, whereas lumbar, sacral, and caudal vertebrae of the tail are derived from the tail bud.

Classic genetic studies have identified >100 mouse mutants with skeletal defects (Lyon and Searle 1989), and this number is increasing with the advent of gene targeting (Brandon et al. 1995), indicating that numerous genes are required for proper development of the axial skeleton. These mutants serve as powerful tools to ana-

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lyze the pathway of axial skeleton development by identifying genes that disrupt early steps, such as gastrulation, and later-acting genes that are required more specifically for vertebral formation.

Vestigial tail (*vt*) is an autosomal recessive mutation that results in viable animals with reduced, kinked tails (Heston 1951; Gruneberg 1957, 1974). Typically, many caudal vertebrae are absent, and those that remain exhibit severe morphological abnormalities. Occasionally presacral vertebrae are missing, and caudal, lumbar, and sacral vertebrae exhibit two centers of ossification rather than a single one in the midline (Heston 1951; Gruneberg 1957). From the analysis of somite-stage embryos it was suggested that these vertebral defects are probably secondary to the reduced recruitment of cells to the caudal somites during somitogenesis (Gruneberg 1974). In addition there were apparent anomalies in the development of the caudal neural tube in the region where somite formation was defective (Gruneberg 1974).

We have examined the possibility that *vt* may be related to *Wnt-3a*, which also maps to Chromosome 11 (Nusse et al. 1991). *Wnt-3a* is a member of a large family of secreted glycoproteins that regulate many distinct aspects of vertebrate and invertebrate development (McMahon et al. 1992; Nusse and Varmus 1992). Expression of *Wnt-3a* is first detected at late primitive streak stages, starting ~7.5 dpc, and continues in the tail bud until at least 9.5 dpc. Gene-targeting studies in the mouse demonstrate that *Wnt-3a* is required for the development of somites and the tail bud. Mice homozygous for a null allele (*Wnt-3a^{neo}*) can develop until 12.5 dpc; however, they lack all somites caudal to somite 9 and fail to form a tail bud. Therefore, the body axis usually terminates between the fore- and hindlimbs (Takada et al. 1994). On the basis of this severe somitic phenotype and the absence of tail bud formation it was suggested that *Wnt-3a* signaling may regulate somite fate and establishment or maintenance of the tail bud (Takada et al. 1994). Here we provide several lines of evidence that the defect in *vt* animals results from the generation of a hypomorphic allele of *Wnt-3a*. In addition, by generating embryos carrying different allelic combinations, thereby progressively lowering the levels of *Wnt-3a* expression, somitogenesis and subsequent axial skeleton development can be arrested at increasingly more anterior positions. These data suggest that *Wnt-3a* is required throughout tail bud development for the elaboration of new somites. Moreover, alteration of the gene dosage suggests that increasing levels of *Wnt-3a* activity are necessary for the development of more posterior somites.

Results

Wnt-3a and *vt* cosegregate on mouse Chromosome 11

The *vt* mutation arose spontaneously in a C57BR stock in 1947 (Heston 1951). The mutation was subsequently crossed to a variety of genetic backgrounds in the course of classic genetic mapping experiments and has been maintained recently with the recessive wavy coat

marker (*wa2*) mutation by backcrossing to a hybrid stock composed of C57BL6/J and C3H/HeJ. Thus, the mutated allele in *vt* mice is derived from the C57BR strain.

Classic genetic mapping localized *vt* relative to several other phenotypic markers on the central portion of Chromosome 11 (Michie 1955b; Searle 1961). To place *vt* relative to molecular markers and to evaluate candidate genes we generated a backcross with a vestigial tailed stock carrying *wa2* and the wild mouse *Mus castaneus*. A range of tail phenotypes were represented among the progeny. Of the 447 progeny collected, 207 were typed as *vt/vt* and 240 as *vt/+* on the basis of tail length and degree of kinking. The average tail length of *vt/+* mice at 3–6 weeks of age was 7.3 ± 0.8 cm ($n=131$) compared with 1.4 ± 1.3 cm ($n=109$) for *vt/vt* mice. The ratio of normal to vestigial-tailed mice did not differ significantly from the expected values for this cross ($\chi^2=2.42$, $df=1$, $P>0.1$). Sixty-seven progeny were scored phenotypically for *vt* and *wa2* and typed for markers spanning the proximal half of Chromosome 11, including *D11Mit1*, *D11Mit22*, *D11Mit5*, and granulocyte macrophage colony-stimulating factor (*Csfgm*). In all cases, the *M. castaneus* alleles were distinguishable from the alleles carried in the vestigial-tailed stock. The location of *vt* and *wa2* relative to these molecular markers was determined by minimization of recombination and haplotype analysis of these and the additional animals. The following gene order and genetic distances were obtained: centromere–*D11Mit1*–15 cM–*wa2*–19 cM–*D11Mit22*–3.6 cM–*Csfgm*–3.5 cM–*vt*–2.2 cM–*D11Mit5* (Fig. 1). Analysis of this backcross provides a basis for correlation of the molecular and classic genetic maps and places *vt* ~5 cM distal to the location predicted in the consensus map (Lossie et al. 1994).

We considered both osteonectin (*Sparc*) and *Wnt-3a* as candidate genes. *Sparc* encodes a secreted protein that is proposed to have a role in bone morphogenesis and somite formation (Mason et al. 1986; Sage and Bornstein 1991; Lane and Sage 1994). Although its precise function is not known, antibody neutralization of osteonectin in *Xenopus* embryos has been shown to disrupt caudal development in an analogous fashion to the backbone truncation and caudal defects observed in *vt/vt* embryos (Purcell et al. 1993). *Sparc* had been mapped previously to the central portion of Chromosome 11 with a 99% probability of being within 3.9 cM of *Csfgm* (Buckwalter et al. 1991). To evaluate *Sparc* as a candidate gene for *vt*, 173 backcross progeny were typed for *Sparc* using a restriction fragment-length protein (RFLP) revealed by Southern blot hybridization. Four recombinants were detected, placing *Sparc* 2.3 ± 1.1 cM proximal to *vt* and unambiguously eliminating it as a candidate gene (Fig. 1).

Wnt-3a encodes a putative signaling molecule essential for caudal development of the tailbud and paraxial mesoderm (Takada et al. 1994) and had been assigned to Chromosome 11 but not localized (Nusse et al. 1991). To localize *Wnt-3a*, the *M. castaneus* backcross segregating *vt* was typed using an RFLP that distinguished the *M. castaneus* allele from *Mus musculus* and *Mus domesticus* alleles of *Wnt-3a*. No recombinants were observed in

Figure 1. Cosegregation of *vt* and *Wnt-3a* in a high-resolution backcross. A map of proximal mouse Chromosome 11 was constructed based on haplotype analysis of markers in a backcross segregating *wa2* and *vt*. The genetic distances between loci are given in centimorgans with estimated standard deviations (Lyon and Searle 1989). No recombinants were detected between *Wnt-3a* and *vt* in 445 of backcross progeny analyzed.

363 progeny. An additional 82 animals were typed using an interval mapping strategy. Animals exhibiting recombination between *D11Mit22* and *D11Mit5* were analyzed further by PCR amplification of a simple sequence length polymorphism (SSLP) in the 3'-untranslated region (UTR) of *Wnt-3a* (see Materials and methods). No recombinants were detected. The lack of crossovers between *vt* and *Wnt-3a* in a total of 445 offspring demonstrates that *vt* and *Wnt-3a* are within 0 ± 0.7 cM of each other (95% confidence level). To date, no genes other than *Wnt-3a* have been mapped to the nonrecombinant region around the *vt* locus. The colocalization of *vt* and *Wnt-3a* provides compelling genetic evidence that a lesion in *Wnt-3a* may be responsible for the *vt* mutation.

Wnt-3a coding sequences are intact in *vt/vt* mice

The gross integrity of *Wnt-3a* was examined by Southern blot analysis using a *Wnt-3a* cDNA probe that contained almost all of the coding region and a portion of the 3' UTR (Roelink and Nusse 1991). Digestion of genomic DNA samples from several different inbred strains of mice with 26 different restriction enzymes revealed RFLPs between C57BL6/J and C57/BR with *StuI* and *SacI* (data not shown). Further RFLP analysis with *StuI* and *SacI* confirmed that *vt/vt* mice contain the C57BR allele of *Wnt-3a*. No differences between *vt/vt* mice and C57BR mice were detected with any of the 26 restriction enzymes tested (data not shown). Therefore, the lesion in

Wnt-3a does not appear to be a gross rearrangement or a large duplication or deletion.

We examined RNA prepared from mutant and normal adult mice and 12.5-dpc embryos by Northern blot using the *Wnt-3a* cDNA probe. *Wnt-3a* mRNA has been detected previously in embryos from 7.5 (Takada et al. 1994) to 12.5 dpc by in situ hybridization (Roelink and Nusse 1991) and in adult lungs by Northern blot analysis (Roelink and Nusse 1991). The sensitivity of our Northern blots was not sufficient to detect *Wnt-3a* mRNA in 12.5-dpc embryos, but the *Wnt-3a* transcripts in adult lung were readily detectable and appeared to be the same size in normal and *vt* mice (data not shown).

The *Wnt-3a* cDNA-coding region was cloned and sequenced from *vt* mice. Two overlapping fragments encompassing 90 bp of 5' UTR, the 1055 bp of coding sequences, and 25 bp of the 3' UTR were sequenced. The *Wnt-3a* cDNA fragments were cloned from three separate RT-PCR amplifications of different preparations of adult lung mRNA. For each fragment, the nucleotide sequence of at least three independent clones was determined. Nucleotide changes not confirmed by independent trials were attributed to *Taq* polymerase errors. Three silent changes were detected between *vt/vt* and the published BALB/c sequence (Roelink and Nusse 1991). At nucleotide 839, a T → C transition was observed, and A → G transitions were noted at nucleotides 932 and 974. These silent changes probably represent polymorphisms specific to the C57BR strain.

In summary, examination of the *Wnt-3a* gene by Southern analysis and the mRNA by Northern and cDNA sequence analysis revealed no signs of the most obvious mutations: gene duplication, deletion, improper splicing, and coding errors. Clearly, *vt* mice have the potential to produce functional *Wnt-3a*. Thus, *vt* may represent a regulatory mutation affecting levels of *Wnt-3a* expression.

Reduced expression of *Wnt-3a* in *vt/vt* embryos

Wnt-3a expression was examined in *+/+*, *+/vt*, and *vt/vt* embryos at 9.5, 10.5, and 11.5 dpc. Previous studies have demonstrated that *Wnt-3a* is expressed in the tail bud at 9.5 dpc (Takada et al. 1994) but it was not clear whether this expression persists throughout the later period of caudal somite formation. Whole-mount in situ hybridization was performed with a minimum of five embryos of each genotype at each stage. In addition, we examined *+/+* embryos that were derived from two different matings, C57BR (the strain on which the *vt* mutation arose) and C57BL6/J × C3H/HeJ (the *F₁* background on which the *vt* mutation is maintained).

At all stages *Wnt-3a* expression was observed in the tail bud of *+/+* and *+/vt* embryos consistent with *Wnt-3a* having a role in late somitogenesis (Fig. 2). In contrast, *Wnt-3a* expression was barely detectable in the tail bud of *vt/vt* embryos even at 9.5 dpc. Expression in the dorsal central nervous system (CNS) of *vt/vt* embryos appeared similar to that of *+/+* and *+/vt* embryos suggesting a preferential loss of *Wnt-3a* expression in the tail bud.

Figure 2. Whole-mount in situ hybridization demonstrates that *Wnt-3a* mRNA is reduced in the tail bud of *vt/vt* embryos. Whole-mount in situ hybridization of a *Wnt-3a* probe (Takada et al. 1994) to 9.5, 10.5, and 11.5 dpc mouse embryos of the following genotypes: $+/+$, $+/vt$, vt/vt . A minimum of five embryos of each genotype were analyzed in a single experiment. Only the tail region is shown for 10.5 and 11.5 dpc embryos. Expression of *Wnt-3a* in the forebrain is indistinguishable at 9.5 dpc among the genotypes (arrows). In contrast, tailbud expression (arrowheads) is greatly reduced in *vt/vt* embryos relative to the levels observed in $+/vt$ and $+/+$ embryos.

Interestingly, the reduction in *Wnt-3a* expression precedes the appearance of an overt loss of somites in *vt/vt* embryos that is first apparent ~10.5–11.0 dpc.

To eliminate the possibility that the uterine environment of *vt/vt* mothers could contribute to differences in fetal expression (Heston 1951), we took advantage of our ability to genotype embryos prior to the appearance of an obvious tail phenotype. Female heterozygotes (*vt/+*) that inherited a normal allele from a C57BL/6J mother were mated with *vt/vt* males. The *vt/vt* and *vt/+* fetuses were distinguished by monitoring for the inheritance of the C57BL/6J allele of *Wnt-3a* using an SSLP (Materials and methods). As expected, the intensity of staining for *Wnt-3a* was reduced considerably in *vt/vt* ($n = 5$) relative to $+/vt$ embryos ($n = 3$) (data not shown). In summary, the *vt* mutation leads to a reduction in *Wnt-3a* expression that precedes the *vt* phenotype. Together, the genetic cosegregation analysis and gene expression studies support the hypothesis that *vt* is a hypomorphic allele of *Wnt-3a*.

Wnt-3a and *vt* are noncomplementary

To confirm this hypothesis, an allelism test was performed between *vt* and *Wnt-3a^{neo}*, a likely null allele generated by gene targeting (Takada et al. 1994). *Wnt-3a^{neo}* homozygotes exhibit embryonic lethality by 12.5 dpc, but heterozygotes are viable (Takada et al. 1994). Genetic analysis has shown that the *vt* lesion is viable with no evidence of lethality (Heston 1951). Females heterozygous for the null allele (*Wnt-3a^{neo}/+*) were mated with *vt/vt* males and progeny were examined at birth. A 50:50 distribution of normal and affected mice would be expected for noncomplementing alleles. However, lack of aberrant tail morphology among the progeny could either be evidence of complementation between *Wnt-*

3a and *vt* or lethality of the compound heterozygotes (*Wnt-3a^{neo}/vt*). Thus, the allelism test was performed with *Wnt-3a^{neo}/+* mice containing the polymorphic C57BL/6J *Wnt-3a⁺* allele, permitting unambiguous genotyping of the progeny.

At birth, 60 progeny had normal tails and 51 had no tails (Figs. 3 and 4B). By χ^2 analysis this distribution does not differ significantly from the 50:50 distribution predicted for noncomplementarity of *vt* and *Wnt-3a* ($P > 0.25$). To confirm that all affected progeny were compound heterozygotes, neonates were genotyped at the *Wnt-3a* locus for the SSLP that distinguishes the *Wnt-3a⁺* allele (C57BL/6) from the embryonic stem-cell-derived *Wnt-3a^{neo}* (129/Sv) and *vt* (C57BR) alleles (Fig. 3). There was a perfect correlation between the phenotype and the genotype determined by the *Wnt-3a* SSLP. The wild-type C57BL/6 allele of *Wnt-3a* was present in all of the neonates with tails but was not present in any of the affected progeny, demonstrating that *Wnt-3a* and *vt* are noncomplementing alleles.

Abnormalities in compound heterozygotes are more extreme than in vt homozygotes

Multiple features of the compound heterozygote phenotype were intermediate in severity between those exhibited by *vt/vt* or *Wnt-3a^{neo}/Wnt-3a^{neo}* mice. The vestigial tail evident in almost all *vt/vt* neonates (Fig. 4A) was completely lacking in compound heterozygotes (Fig. 4B, center). The most severely affected compound heterozygotes had smaller bodies, hindlimb paralysis, and spina bifida of the posterior neural tube (Fig. 4B, right). Five litters from the allelism test cross were sequestered to assess postnatal lethality. Approximately 50% of the compound heterozygotes lived longer than 4 weeks (12 of 27), compared with 90% of *vt/+* littermates (19 of 21),

Figure 3. Noncomplementation of *Wnt-3a* and *vt*. Male *vt/vt* mice were mated with *Wnt-3a*^{neo/+} females, and the phenotype (tail or no tail) of the progeny was assigned at birth. The genotype at the *Wnt-3a* locus was determined by PCR amplification of a polymorphic simple sequence repeat in the 3' UTR of *Wnt-3a*. A similar pattern of fragments was produced using genomic DNA prepared from 129/SvJ and C57BR/cdJ mouse strains (lanes 1,3), and a larger product was obtained from C57BL6/J genomic DNA (lane 2). The *vt/vt* parent (lane 4) is homozygous for the smaller fragment, which is identical in size to the product amplified from C57BR/cdJ genomic DNA, the strain on which *vt/vt* arose. The *Wnt-3a*^{neo} allele was generated in embryonic stem cells derived from the 129/SvJ mouse strain. Chimeric mice were mated to C57BL6/J mice to produce *Wnt-3a*^{neo/+} heterozygotes and maintained by crossing with C57BL6/J (Takada et al. 1994). *Wnt-3a*^{neo/+} females (lane 5) are heterozygous for the embryonic stem cell-derived *Wnt3a*^{neo} allele (lower band) and the larger C57BL6/J derived *Wnt3a*⁺ allele. Analysis of four progeny of the allelism test is illustrated (lanes 6–9). PCR amplification of genomic DNA revealed the presence of the C57BL6/J *Wnt3a*⁺ allele in progeny with tails (half-filled diamonds, lanes 7,9) but not in the tailless progeny (solid diamonds, lanes 6,8). The results are summarized at the bottom. Fifty-one newborns with tails were genotyped as *vt/+*, and 29 newborns without tails were genotyped as *Wnt-3a*^{neo/vt} compound heterozygotes. No genotyping information was available for the 31 progeny classified as “not determined” because they died before tissues were collected for DNA preparation or the DNA was insufficient for analysis.

demonstrating a significant reduction in the viability of compound heterozygotes ($P < 0.001$). For most compound heterozygotes the cause of death was not clear, but in several cases an accumulation of urine was observed in kidneys and ureters, and intestines were hyperextended,

suggesting a defect in excretory system function. Defects in the second through fourth sacral vertebrae are commonly associated with neurogenic bladder in humans (Stark 1977).

Skeletal defects in compound heterozygotes extended more anteriorly than those of *vt/vt* mice (Figs. 4 and 5). Ten skeletons from *vt/+* neonates were analyzed, and all showed normal structure and number of vertebrae through the cervical, thoracic, lumbar, sacral, and caudal regions. Fourteen skeletons of *vt/vt* neonates revealed normal development through the sacral region, but the number of caudal vertebrae with normal morphology was only 2–15 compared with 18–25 observed in *vt/+* mice (Fig. 5). In addition, the most caudal vertebrae in *vt/vt* neonates exhibit abnormal centers of ossification. These observations are consistent with published reports of the *vt/vt* phenotype (Heston 1951; Gruneberg 1957), except that no sacral defects were observed in this sampling of our stock (Fig. 5). The skeletal phenotype of 12 compound heterozygotes was also variable (Fig. 5), but in all cases the defects were much more severe than those observed in *vt/vt* mice. None of the *Wnt-3a*^{neo/vt} mice had a full complement of lumbar vertebrae, and no normal sacral vertebrae were observed in individuals of this group. The most severely affected compound heterozygotes exhibited a reduced number of thoracic vertebrae and deformities as far anterior as the seventh thoracic vertebra (Fig. 4F). The results of this phenotypic analysis are summarized (Fig. 5). Whereas the skeletal phenotypes observed for each genotype were variable, no overlap was observed between genotypes.

To confirm that the skeletal phenotype of neonates is an accurate indicator of early developmental events, we examined the formation of somites at 10.5 and 11.5 dpc with the marker *Mox-1*. *Mox-1* is first expressed in the presomitic mesoderm, continues into newly formed epithelial somites (8.0–9.5 dpc), and persists as the epithelial somites differentiate to the sclerotome, dermatome, and myotome (9.5–11.5 dpc) (Candia et al. 1992). In the *Wnt-3a*^{neo}/*Wnt-3a*^{neo} mutants, as reported previously (Takada et al. 1994), somites with normal *Mox-1* expression only extend to the level of the forelimb bud (Fig. 6D). Somite development in compound heterozygotes (*Wnt-3a*^{neo/vt}) extended just posterior of the hindlimb bud (hindlimb ends at somite 28, lumbar region). Some nonepithelial *Mox-1* expression was observed for a short distance caudal to this position, and then expression terminated more rostral to the tip of the tail than in *+/+* embryos (Fig. 6, cf. A and C). Skeletal analysis of *Wnt-3a*^{neo/vt} neonates revealed morphological abnormalities rostral to the last somites observed to express *Mox-1* in embryos (Fig. 5), indicating that the expression of *Mox-1* is not sufficient for normal somite function. At 10.5 dpc the *vt/vt* embryos showed similar patterns of *Mox-1* expression to *+/+* embryos, indicating that somite formation extending to the precursors of the anterior tail vertebrae (initiated at somite 35) was normal in *vt/vt* embryos (Fig. 6B). However, by 11.5 dpc, clear differences were apparent in somite number and *Mox-1* expression in the caudal region of *vt/vt* embryos (Fig. 6F) relative to

Figure 4. (A) Skeletal abnormalities of *vt/vt* mice are less severe than those observed in *Wnt3a^{neo}/vt* compound heterozygotes. The skeletal morphology was analyzed by staining bone with alizarin red and cartilage with alcian blue. In A, analysis of a normal day 2 neonate (*vt/+*, far left) and four *vt/vt* littermates reveals the range of deformed tails characteristic of *vt/vt* mice. Progeny of the allelism test (B) included a normal neonate (far left) and two compound heterozygotes (*Wnt-3a^{neo}/vt*) with no visible tail. Most compound heterozygotes had normal hindlimb reflexes (middle), but the most severely affected *Wnt-3a^{neo}/vt* mice had hindlimb paralysis and spina bifida (far right). The skeletons of *vt/+* neonates (C) were compared with *vt/vt* (D) and *Wnt-3a^{neo}/vt* skeletons (E,F). The position of the most posterior normal vertebra is indicated with a solid arrowhead. The last thoracic (T13), the last lumbar (L6), the first sacral (S1), and the first caudal (Cd 1) vertebra are indicated as landmarks (arrows in C–E). Vertebral defects, including abnormal centers of ossification, malformations, and fusions, were evident in the caudal region of all *vt/vt* mice (D; Cd 3 = normal, arrowhead), in the lumbar region of a *Wnt-3a^{neo}/vt* mouse with an intermediate phenotype (E; L1 = normal, arrowhead), and the thoracic region of the most severely affected *Wnt-3a^{neo}/vt* mice (F; T6 = normal, arrowhead).

wild-type littermates (Fig. 6E). Thus, there is a good correlation between the axial position at which somite formation is disrupted and the later arising skeletal phenotypes in the different allelic combinations, indicating that the loss or disruption of vertebrae is a primary consequence of the failure to form normal somites.

Discussion

The data presented here are consistent with the hypothesis that *vt* is a hypomorphic allele of *Wnt-3a*. First, no recombination occurred between *Wnt-3a* and *vt* in 445 offspring from the mapping crosses, placing *Wnt-3a* and *vt* within 0.7 cM on Chromosome 11. In addition, a null mutation in *Wnt-3a* failed to complement *vt*. Compound heterozygotes have a strikingly severe failure of paraxial

mesoderm formation and lethal phenotype. In *vt/vt* mice the tail is shortened because of the absence of caudal vertebrae and kinked because of vertebral abnormalities, reflecting an underlying failure of caudal somite formation. The phenotype of *vt/vt* embryos is therefore consistent with two facets of *Wnt-3a* expression: the observation that *Wnt-3a* regulates somite formation at earlier stages (7.5 dpc), and the persistent expression of *Wnt-3a* in the tail bud to at least 12.5 dpc (S. Takada and A. McMahon, unpubl). This indicates that *Wnt-3a* might also regulate the formation of tail bud-derived somites. Furthermore, *Wnt-3a* expression in the tail bud of *vt/vt* embryos is reduced prior to the appearance of an overt phenotype in *vt/vt* embryos. Finally, *Wnt-3a* encodes a secreted protein and would be expected to act non-cell autonomously. Genetic studies of the *Drosophila* rela-

Figure 5. The skeletal abnormalities of vestigial-tailed homozygotes occur in more posterior positions than in *Wnt3a^{neo}/vt* mice. The most posterior position at which normal vertebrae were apparent in an individual neonatal skeleton preparation is designated for mice genotyped as *Wnt3a^{neo}/vt* (open circles), *vt/vt* (shaded circles) and *vt/+* (solid circles). Asterisks denote the skeletons illustrated in Fig. 4.

tive *wingless* [*wg* (Morata and Lawrence 1977)] and of mouse *Wnt-1* (G. Vassileva, K. Stark, J.A. McMahon, and A.P. McMahon, in prep.) both demonstrate non-cell autonomy. Interestingly, analysis of aggregation chimaeras has also demonstrated that *vt* is non-cell autonomous, consistent with a lesion in a secreted protein (Gruneberg and McLaren 1972).

It seems most likely that the reduced levels of *Wnt-3a* expression in *vt/vt* embryos reflect an underlying quantitative defect in *Wnt-3a* transcription, although no genomic rearrangements that might cause this were apparent. Moreover, as expression in the dorsal spinal cord and brain appears normal at 9.5 dpc, loss of *Wnt-3a* expression may result from the disruption of a tail bud-specific regulatory region. However, although there is no evidence for differential splicing in either embryos or in adult lung, we cannot rule out a mutation that would affect efficiency of splicing or polyadenylation, or a mutation in the 3' UTR that would affect RNA stability and consequently reduce steady-state levels. Coincidentally, molecular lesions associated with three hypomorphic alleles of *wg* have been detected recently, 3' of the gene

(van den Heuvel et al. 1993; Couso and Arias 1994) in a putative regulatory region.

If the *vt* lesion is a point mutation in an enhancer region, it may be difficult to locate. Potential regulatory mutations can be quite distant from the promoter. For example, two regulatory mutations of *Steel* (*Sl*), *Sl^{pan}* and *Sl^{con}*, involve DNA rearrangements located >100 kb upstream of the coding sequences (Bedell et al. 1995). Nothing is known about the transcriptional regulation of

Figure 6. The axial level at which somite formation terminates correlates with the levels of *Wnt-3a* activity in different allelic combinations. Somite formation in 10.5 dpc (A–D) and 11.5 dpc (E–F) embryos was analyzed by examining expression of the somite marker, *Mox-1*. Embryos shown are as follows: +/+ (A,E), *vt/vt* (B,F), *Wnt-3a^{neo}/vt* (C), and *Wnt-3a^{neo}/Wnt-3a^{neo}* (D). Somite formation is visibly disrupted in *Wnt-3a^{neo}/vt* (C) and *Wnt-3a^{neo}/Wnt-3a^{neo}* (D) embryos at 10.5 dpc but is normal in *vt/vt* embryos at this time. However, caudal somite formation fails to occur in *vt/vt* embryos by 11.5 dpc (F). Arrows mark the position at which *Mox-1* expression terminates.

Wnt-3a, and the genomic structure has not been reported. Therefore, a precise molecular description of the *vt* mutation is beyond the scope of the present study, but it is likely to provide an important insight into the regulation of signaling in the vertebrate tail bud. Until the molecular basis for the lesion is identified, we cannot exclude the possibility that *vt* results from a lesion in another as yet unidentified gene within 0.7 cM of *Wnt-3a* that acts in the *Wnt-3a* pathway. An example of such nonallelic noncomplementation has been observed between the *Brachyury*, or *T* locus, and a neighboring gene (Rennebeck et al. 1995)

Although there are only two mutant alleles of *Wnt-3a*, *Wnt-3a^{neo}* and *vt*, the functional consequences of five different *Wnt-3a* levels can be observed by comparing the phenotype of *Wnt-3a^{neo}* homozygotes, *Wnt-3a^{neo}/vt* compound heterozygotes, *vt* homozygotes, and the *Wnt-3a^{neo}/+* and *vt/+* heterozygotes (Fig. 7). Analysis of this *Wnt-3a* allelic series demonstrates a requirement for increasing *Wnt-3a* dosage for the elaboration of progressively more caudal paraxial mesoderm derivatives. Homozygotes for the null allele exhibit no somite development posterior to somite 9. Because most of these

embryos die by 12.5 dpc, prior to vertebral formation, data on the skeleton are limited. However, somite 9 gives rise to the fifth and sixth cervical vertebrae; therefore, it is likely that the vertebral formation would usually terminate in the neck (Fig. 7). In *Wnt3a^{neo}/vt* compound heterozygotes, the small amount of *Wnt-3a* produced from one *vt* allele is sufficient for prenatal viability and normal development of most thoracic vertebrae (Fig. 7). Higher levels of *Wnt-3a* produced in *vt/vt* individuals completely rescue the postnatal viability and are sufficient for normal development of the lumbar and sacral regions, though not for the posterior tail vertebrae (Fig. 7). Finally, there is evidence that the reduced dosage of *Wnt-3a* in *vt/+* mice is not always sufficient for the formation of all vertebrae, sometimes resulting in fewer caudal vertebrae and morphological irregularities that produce tail kinks (Gruneberg and McLaren 1972).

Genetic background contributes to the variation in severity of phenotype that we have observed within each genotype class; however, differences between groups are attributable to gene dosage. When *vt* was placed on the 129/SvJ background, the most severe individual showed no caudal vertebrae development, but the sacral verte-

Figure 7. Relationship between *Wnt-3a* dosage and formation of axial skeleton. Lower doses of *Wnt-3a* cause more anterior defects in the skeleton. The vertebrae of the axial skeleton are depicted diagrammatically (x-axis) with the approximate day of embryonic development at which each region develops. The genotype and estimated levels of *Wnt-3a* activity for the allelic series are indicated (y-axis). Bars depict the relative development of the skeleton based on observation of the *Wnt-3a^{neo}/Wnt-3a^{neo}* embryos (Takada et al. 1994), skeletal preparations of *vt/vt* and *Wnt-3a^{neo}/vt* neonates, and the observed tail morphology of adult *Wnt-3a^{neo}/+* mice and *vt/+* backcross progeny. The bars suggest the phenotypic range of vertebral development for each genotype. The actual levels of *Wnt-3a* expression from the *vt* allele are not known. Thus, the position of mice carrying *vt* alleles on the *Wnt-3a* activity scale is arbitrarily assigned at a position lower than that of the wild-type *Wnt-3a* allele but greater than that of the *Wnt-3a* null allele.

brae were intact. Therefore the severity in the compound heterozygotes compared with *vt* homozygotes is attributable to gene dosage and cannot be attributed to background effects. On an (C57BL6/J $\times$ 129/Sv) F₁ hybrid background, *Wnt-3a^{neo}* heterozygotes exhibit a low frequency (10% of heterozygotes) of terminal tail kinks. On a 129/SvJ inbred background the penetrance is much higher and 80% of heterozygotes exhibit this phenotype (S. Takada, M. Hammerschmidt, and A.P. McMahon, unpubl.). Similarly, occasional tail kinks have been observed in *vt/+* mice (Michie 1955a; Gruneberg and McLaren 1972), and we observed tail kinks in 1%–2% of the *vt/+* progeny of the *M. castaneus* backcross. Thus, on the appropriate genetic backgrounds *Wnt-3a* is haploinsufficient. More than 50% of normal activity is required for normal morphological development of the caudal-most vertebrae (Fig. 7). Interestingly, mutants in *Brachyury*, a transcriptional regulator that is also expressed in mesodermal precursors in the tail bud, exhibit haploinsufficiency and an allelic series with a similar increased requirement along the rostral to caudal axis (MacMurray and Shin 1988; Stott et al. 1993).

The allelic series of *Wnt-3a* in mice resembles the series of *wg* alleles in *Drosophila*. Nine null alleles of *wg* are embryonic lethal, two hypomorphic alleles are lethal at the pupal stage (van den Heuvel et al. 1993), and the least severe hypomorphic allele results in viable adults that lack one or both wings (Sharma and Chopra 1976). The range of viability observed in the *wg* allelic series corresponds to the embryonic lethal, perinatal lethal, and adult viable phenotypes observed in mice with varied amounts of *Wnt-3a*. The example provided by *Wnt-3a* suggests that the use of gene targeting to generate hypomorphic alleles of other mammalian *Wnt* genes could be enlightening.

What is the role of *Wnt-3a* and what is the significance of the dosage requirements? Two phenotypic features of the *vt* hypomorph and *Wnt-3a^{neo}/vt* heterozygotes bear a striking resemblance to the phenotype of null mutants. First, all genetic combinations result in axial truncation albeit at different rostral–caudal positions. Thus, the process of axial progression is dependent on *Wnt-3a* activity from early somite stages until its completion. Earlier work indicates that this reflects a requirement for *Wnt-3a* for specification or maintenance of mesodermal precursors in the tail bud. Second, somites are absent for a considerable distance rostral to the position at which the axis truncates. Therefore, the axis continues to elongate but somites do not form. Again the position is dependent on the genetic combination and, consequently, the levels of *Wnt-3a* expression. Thus, *Wnt-3a* may have a direct role in the development of somitic mesoderm.

The dosage requirements are open to several different interpretations. For example, there may be an absolute requirement for increasing levels of *Wnt-3a* activity as development proceeds. In one scenario, the levels of *Wnt-3a* activity are only relevant in so much as they satisfy the demand at a given time for somite formation and tail bud development. Thus, *Wnt-3a* acts in exactly the same way at all stages; however, the system de-

mands increased *Wnt-3a* activity at later stages than at earlier times. Alternatively, the differing requirement for *Wnt-3a* activity at different axial levels may relate to the distinct fates of somites forming at these levels. Therefore, whereas, a certain level may be sufficient for a thoracic vertebral fate, higher levels may be necessary to generate caudal vertebrae. Furthermore, it is possible that a constant threshold of *Wnt-3a* signal is actually required, but a gradual decrease in the transcription of *Wnt-3a* occurs in the tail bud. At early stages, *Wnt-3a* expression may be in considerable excess of that required to achieve the threshold levels of *Wnt-3a* protein. In this case, if steady-state levels of transcript decrease, two normal alleles of *Wnt-3a* may only produce a small excess of *Wnt-3a*. Consequently, loss of activity in either one or both alleles would lead to subthreshold levels of *Wnt-3a*, causing the arrest of somite formation and axial elongation. Clearly, additional experiments will be required to determine the significance of *Wnt-3a* dosage requirements. However, these studies underscore the importance of *Wnt-3a* signaling for mesodermal development in the mouse, and presumably other vertebrates, and provide suggestive evidence that the levels of *Wnt* signals may be important for different developmental outcomes along the rostral–caudal axis.

In humans, caudal defects including spina bifida, caudal regression syndrome, and sirenomelia bear many characteristics of the *Wnt-3a* allelic series, including various defects of the lower neural tube, gastrointestinal tract and genitourinary tracts, fusion of the lower limbs, and reduced number of presacral vertebrae (Smith 1982; Buyce 1990; Takada et al. 1994). These devastating birth defects have an incidence of ~ 1 in 10,000 and are one of the most common causes of stillbirth and early neonatal death (Pauli 1994). Whereas the etiology of these lower mesodermal defects is not well understood, it is clear that both genetic and environmental factors contribute to the risk (Reece and Hobbins 1986; Werler et al. 1993). It will be of considerable importance to assess the contribution of human *WNT* gene expression to the genetic risk for these syndromes.

Materials and methods

Mice

The following mouse stocks were purchased from the Jackson Laboratory, Bar Harbor, ME: *M. musculus castaneus* (CASA/Rk, F₄₀), C57BL6/J, C57BR/cdJ, 129/SvJ, and *vestigial tail* (B6C3H-a/A-*wa2 vt*). A stock carrying the *vt* mutation, referred to herein as VT, was maintained at The University of Michigan (Ann Arbor) since 1991 by intercrossing without further backcrossing to B6C3H. Mice heterozygous for a null allele of *Wnt-3a* (*Wnt-3a^{neo}/+*) have been described previously (Takada et al. 1994).

Genetic mapping

Progeny of the (VT-*vt/vt* $\times$ *M. castaneus* F₁ $\times$ VT-*vt/vt*) backcross were scored at 1–5 weeks of age for the presence or absence of the characteristic *vt/vt* tail phenotype and the first coat of wavy hair and curly whiskers evident in *wa2* homozygotes. Genomic

DNA was prepared from backcross progeny (liver, kidney, lung, spleen, or tail) by proteinase K digestion followed by a high salt extraction [Miller et al. 1988]. SSLPs were detected by PCR amplification of genomic DNA samples. Primers specific for *D11Mit1*, *D11Mit5*, *D11Mit22*, and *Csfgm* have been described [Love et al. 1990]. Amplification of *M. castaneus* and *vt/vt* genomic DNA samples produced products of 120 and 160 bp for *D11Mit1* primers, 130 and 200 bp for *D11Mit5*, and 270 and 240 bp for *D11Mit22*, respectively. The *D11Mit1*, *D11Mit5*, and *D11Mit22* amplification products were separated by electrophoresis on 2% agarose/1% NuSieve gels in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA at pH 8.0) and visualized by ethidium bromide staining. The *Csfgm* SSLP was detected by PCR amplification in the presence of [³²P]dCTP, the products were separated by electrophoresis on 6% polyacrylamide gels, and visualized by autoradiography.

Osteonectin (*Sparc*) and *Wnt-3a* were mapped using RFLPs detected by Southern blot hybridization. The *SPARC* probe (pC33) was a 1.1-kb fragment of mouse osteonectin genomic DNA [kindly provided by Dr. M. Young, National Institute of Dental Research, Bethesda, MD (Holland et al. 1987)]. The *Wnt-3a* probe was a mouse cDNA (p9A) isolated from an 8.5-day embryo mouse library and provided by Dr. Roel Nusse (Stanford University, CA) [Roelink and Nusse 1991; Takada et al. 1994]. The probe spans nucleotides 196–1451, including 267 bp of the 3' UTR and the entire coding region except 67 bp at the 5' end [Roelink and Nusse 1991]. Probes were prepared by radiolabeling purified DNA fragments with [³²P]dCTP (NEN) nucleotide by the random hexamer method [Feinberg and Vogelstein 1982]. Hybridization of filters containing genomic DNA digested with *Bam*HI with the *SPARC* probe detected a *CASA/Rk*-specific fragment of 3.1 kb [Buckwalter et al. 1991] and a *VT*-specific fragment of 2.5 kb. The *Wnt-3a* cDNA probe detected an 8.8-kb *CASA/Rk* specific fragment and a 5.2-kb *VT*-specific fragment in genomic DNA digested with *Bgl*II.

Haplotype analysis and minimization of crossover frequency was used to establish gene order. Genetic distances are reported in centimorgans and the estimated standard error was calculated as the square root of the variance. The variance is equal to $p(1-p)/n$, where p is the recombinant fraction and n is the number of animals tested. When no recombinants were observed between two markers, a 95% confidence interval was calculated using the formula $P = (1-r)^n$, where P is the probability of a particular event (0.05 for a 95% confidence interval), r is the maximum distance between the two loci, and n is the number of meioses scored [Friedman et al. 1991].

Detection of *Wnt-3a* by SSLP

The *Wnt-3a* cDNA sequence was analyzed for the presence of simple sequence repeats using a Pustell DNA matrix homology algorithm [MacVector, IBI/Kodak] [Roelink and Nusse 1991]. A thymidine-rich region (T₄CT₁₃CT₄CT₃) was detected at position 2680–2707 in the 3' UTR of *Wnt-3a*. Oligonucleotide primers were designed that flank this region: 5' → 3' forward, GAC-TGACTGTCTTCTGCCTGG; reverse, ACTTTCCCCATTAT-TTGAAACA. Ten-microliter reactions, containing 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotides, 0.2 pmole of each primer, and 0.5 μl of [^α-³²P]dCTP (NEN), were overlaid with mineral oil and denatured for 1 min at 94°C, amplified for 35 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C), followed by a final elongation step (10 min at 72°C). DNA quantities between 40 and 700 ng were amplified successfully. Reactions were combined 1:1 with loading dye, and 5 μl (25% of the PCR reaction) was analyzed by electrophoresis on 6% acrylamide gels with 8.3 M urea and exposure to X-ray film [Kodak

XAR-5) for 4 hr. The fragment amplified from C57BL6/J genomic DNA migrated slower than the amplified products from VT, C57BR/cdJ, and 129/SvJ DNA. The size of this fragment predicted from the BALB/c sequence is 178 bp [Roelink and Nusse 1991].

Sequence analysis of *Wnt-3a* cDNA

Total RNA was prepared from adult lungs using a single-step method [Chomczynski and Sacchi 1987], and the sample was enriched for polyadenylated mRNA by chromatography on poly(dT)–cellulose [Sambrook et al. 1989]. The *Wnt-3a* cDNA was amplified in two overlapping fragments by RT-PCR. Primers designed from the published sequence amplified nucleotides 39–639 (forward, GGTCTAGACACTGCAGCCGCGCCACC-TCCCAGG; reverse, CCGGATCCCGGCAAACCTCCCGAG-AGACCATTCC) and nucleotides 540–1211 (forward, GGTC-TAGATGCAGCAGCCGCTCCAGGGCTCCC; reverse, C-CGGATCCTGCTCCCGTGTAGGAGCTCTCC) [Roelink and Nusse 1991]. The primers included *Xba*I and *Bam*HI recognition sites to simplify cloning the fragments into pGEM-3z (Promega). After digestion with *Xba*I and *Bam*HI, RT-PCR products were separated by electrophoresis on 1% NuSieve agarose, excised, and ligated into pGEM-3z in the presence of the agarose. The ligation mixture was used to transform DH5α cells by electroporation, and colonies were screened on Luria–Bertani medium (LB) plates containing ampicillin (Sigma) and X-gal (BMB). Plasmids were purified by CsCl gradient centrifugation, and the DNA sequence determined using Sequenase, version 2.0 [U.S. Biochemical] and the following pUC sequencing primers (BMB): forward, 5' → 3' GTAAAACGACGGCCAGT; reverse, 5' → 3' CAGGAAACAGCTATGAC.

Whole-mount *in situ* hybridization

Female *vt/+* mice generated by crossing *vt* homozygotes with C57BL6/J mice, were caged with *vt/vt* males and observed daily for the presence of copulation plugs (day 0.5). At 9.5 and 10.5 dpc embryos were removed and processed individually for *in situ* hybridization [Wilkinson 1992]. The corresponding yolk sacs were reserved for genotype analysis. Individual embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated in graded methanols, and stored in 100% methanol at –20°C. Genomic DNA was isolated from yolk sacs [Miller et al. 1988] and resuspended in 20 μl of sterile water. PCR reactions containing 1 μl of DNA were carried out with primers that detect the *Wnt-3a* SSLP. Embryos with identical *Wnt-3a* genotype were pooled from two to four litters and analyzed by *in situ* hybridization with *Wnt-3a* sense and antisense riboprobes. In addition, litters of entirely one genotype were generated by the following matings: *vt/vt* × *vt/vt* and *vt/vt* × C57BR or B6C3H F₁.

The *Wnt-3a* antisense riboprobe, spanning nucleotides 222–1451, was prepared by *Sca*I digestion of the template p15A [Roelink and Nusse 1991] and transcription with Sp6 polymerase (Promega) using a digoxigenin-labeling kit (BMB). As a negative control, a *Wnt-3a* sense probe corresponding to nucleotides 1–1451 was prepared by digestion with *Bam*HI and incubation with T₇ polymerase (Promega). Preparation of the *Mox-1* probe [Candia et al. 1992] has been described previously [Takada et al. 1994]. Conditions for hybridization have also been described previously [Wilkinson 1992].

Skeleton preparations

Animals were collected 1–3 days after birth and killed by ether inhalation. Skin and viscera were removed, and the carcass was

fixed in 95% ethanol overnight. Samples were placed in alcian blue (Sigma) for 2 days (1 ml of 0.3% alcian blue in 70% ethanol, 2 ml of acetic acid, 8 ml of 95% ethanol), dehydrated in 95% ethanol for 5 days, and counterstained with alizarin red (Sigma) (0.25 ml of 0.1% alizarin red in 95% ethanol per 10 ml of 1% KOH). Specimens were placed in 1% KOH for 6 hr, and cleared by incubation in 20%, 50%, and 80% glycerol in a stepwise manner. Destaining times for the 50% and 80% glycerol steps varied from 1 day to >2 weeks, depending on the amount of residual stain. Skeletons were maintained in 100% glycerol.

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