

## Increased DNA Methylation at the *AXIN1* Gene in a Monozygotic Twin from a Pair Discordant for a Caudal Duplication Anomaly

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The *AXIN1* gene has been implicated in caudal duplication anomalies. Its coding region was sequenced in both members of a monozygotic (MZ) twin pair discordant for a caudal duplication anomaly, but no mutation was found. Using bisulfite sequencing, we examined methylation at the promoter region of the *AXIN1* gene in these twins and in twin and age-matched singleton controls. Methylation of the promoter region in peripheral blood mononucleated cells was variable among individuals, including MZ pairs. In the MZ pair discordant for the caudal duplication, this region of the affected twin was significantly more methylated than that of the unaffected twin ( $P < .0001$ ), which was significantly more methylated than those of the controls ( $P = .02$ ). We have confirmed that this CpG island does function as a promoter *in vitro* and that its activity is inversely proportional to the extent of methylation. This finding raises the possibility that hypermethylation of the *AXIN1* promoter, by mechanisms as yet undetermined, is associated with the malformation. This case may be paradigmatic for some cases of MZ discordance.

The high rate of discordance in MZ pairs for most complex diseases is a continuing puzzle. Vague statements that “the environment” is responsible for this discordance are seldom backed up by data, despite often huge investments in searching for the etiologic agents<sup>1</sup>; smoking as a risk factor for lung cancer and heart disease is a rare exception.<sup>2</sup> In recent years, there has been increasing interest in the possibility that stochastically or environmentally triggered differences in the epigenetic status of key genes may be responsible for some MZ discordance and, indeed, for much of the burden of complex disease.<sup>3,4</sup> An obstacle to the testing of this hypothesis is that, for most complex traits, we still have very few identified genes whose methylation status can be investigated and, where we do, the pertinent tissue may be inaccessible for methylation studies on living subjects. Until such genes for common diseases become known, therefore, it is worth investigating cases of MZ discordance for rare diseases in which the causal gene(s) are known or strongly suspected.

In 2002, a pair of female MZ twins were described as discordant for caudal duplication anomaly (MIM 607864), in which one twin had a duplication of the distal spine and other organs as well as a tumor in the lumbosacral region and spina bifida.<sup>5</sup> Caudal duplication anomaly is sporadic and can involve multiple congenital anomalies, but the hallmark is duplication of organs in the caudal region, such as the distal spine.<sup>6</sup> This spinal duplication is similar to that seen in *Axin*<sup>Fu</sup> mice, which carry a mutation at the *Axin* locus.<sup>7</sup> Highly penetrant mice display

bifurcating tails as a result of caudal duplication in the distal region. *Axin* encodes an inhibitor of the Wnt-signaling pathway and has been shown to regulate embryonic axis formation in mouse and in *Xenopus*.<sup>8</sup> Suppression of wild-type *Axin* in *Xenopus* embryos results in the duplication of the body axis. The role of *Axin* in caudal duplication anomaly in humans is not known, but it remains the strongest candidate. Sequence analysis of the *AXIN1* (MIM 603816) coding region in the twin with caudal duplication syndrome revealed no pathogenic mutations, although a rare missense variant was noticed in both twins and in the father.<sup>5</sup> Clearly, this missense variant alone cannot account for the MZ discordance. We thought it possible that *AXIN1* inactivation may have occurred via an epigenetic process.

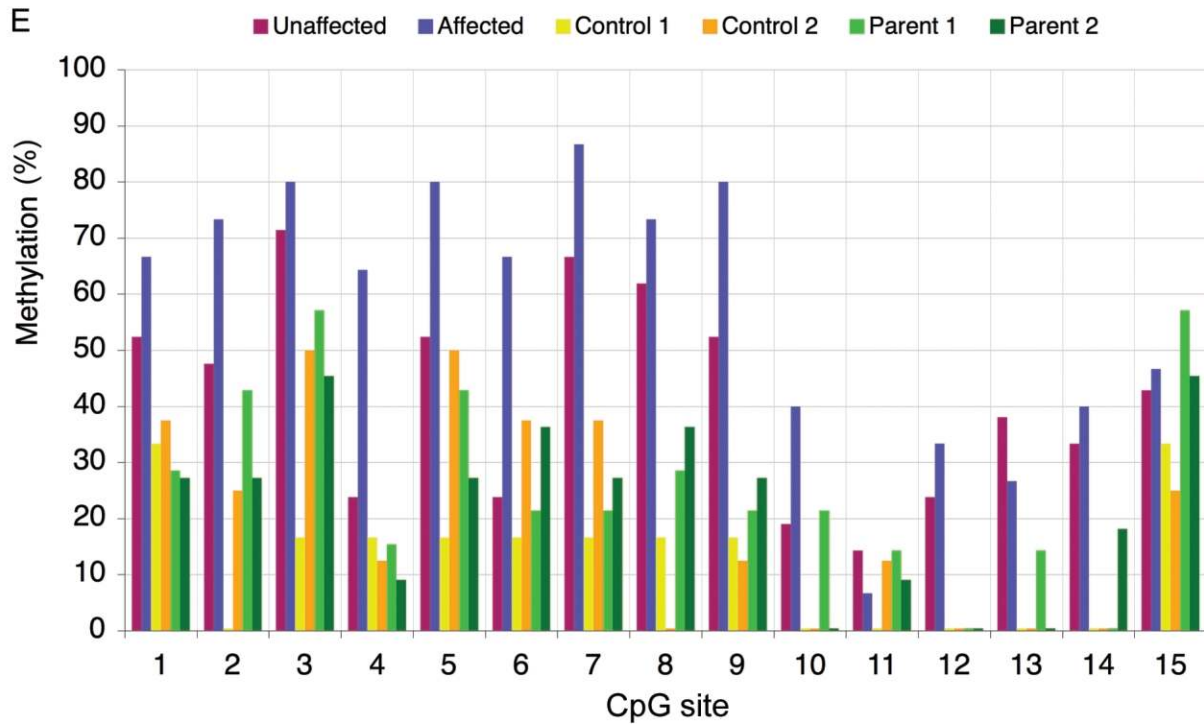
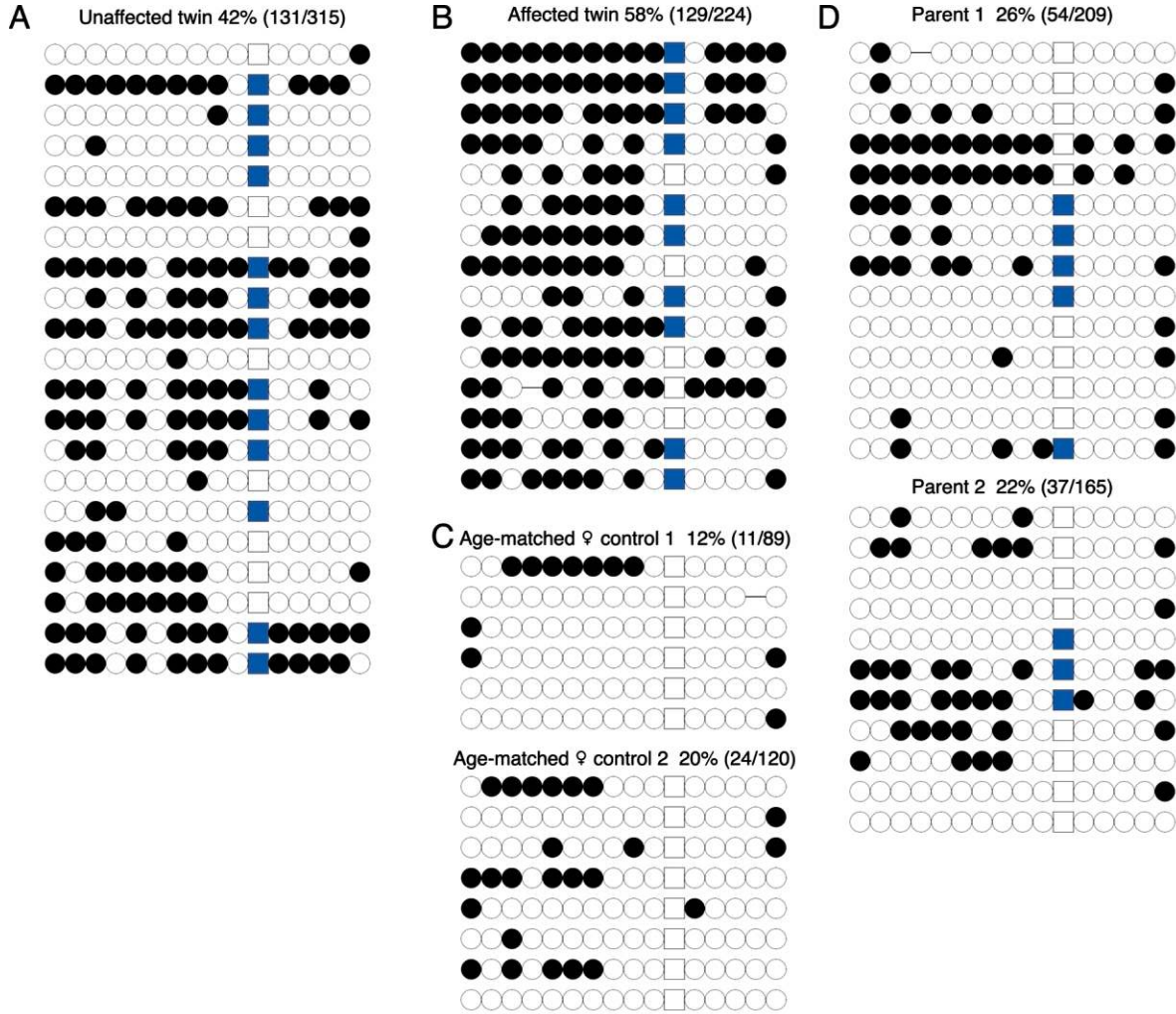
In mammals, methylation of DNA occurs at cytosine residues, mainly at CpG dinucleotides. There are stretches of DNA, CpG islands, that are rich in CpG and are often found at promoter regions of genes. CpG islands are normally unmethylated, but there is now evidence that a small proportion of these are methylated in some tissues.<sup>9,10</sup> Aberrant *de novo* methylation of normally unmethylated CpG islands in humans and mice is associated with transcriptional silencing.<sup>11</sup> For example, the penetrance and severity of the abnormal tail phenotype in inbred—and, therefore, isogenic—*Axin*<sup>Fu</sup> mice correlate with specific patterns of DNA methylation at the *Axin* locus.<sup>12</sup> Here, we report the careful examination of cytosine methylation at the promoter and intergenic regions of human

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*AXIN1* in the MZ twin pair discordant for caudal duplication anomaly, in their parents, and in several controls.

The probands were reported to be monozygotic and probably monoamniotic (H. Kroes, personal communication). Peripheral blood mononucleated cell (PBMC) DNA was collected from them at age 7 mo. Full blood counts taken at the same time from both were unremarkable. Methylation profiles may change slightly with age,<sup>13</sup> so, for age-matched controls, we obtained PBMC DNA from two female hospital patients, the first aged 5 mo with a congenital malformation of the heart and trachea, and the second aged 11 mo with viral myocarditis. PBMC DNA from a further seven adult controls was also examined.

MZ twinning is itself a duplication event, so we were keen to ensure that any abnormalities of methylation of the *AXIN1* gene were specific to the malformation and not inherent to MZ twinning. We obtained PBMC DNA from nine MZ pairs aged 12–14 years at the time of collection, including eight sets of males and one set of females. These pairs were selected from a larger study<sup>14</sup> as being probably dichorionic because the mother had reported two separate placentas at birth. It has been suggested that monozygotic pairs exchange hematopoietic stem cells in utero, making cotwins' blood cell profiles (and perhaps methylation status) more similar than they might otherwise be. The extent of this alleged problem has never been quantified, but, to the extent that it exists, the monozygosity of our proband pair would make our search for methylation differences between cotwins conservative. Monozygosity of all pairs, including the proband pair, was confirmed by typing them with the use of the ABI Profiler Plus set of nine highly polymorphic microsatellite markers and the amelogenin sex marker, yielding a probability of dizygosity, given concordance of  $<10^{-4}$ .

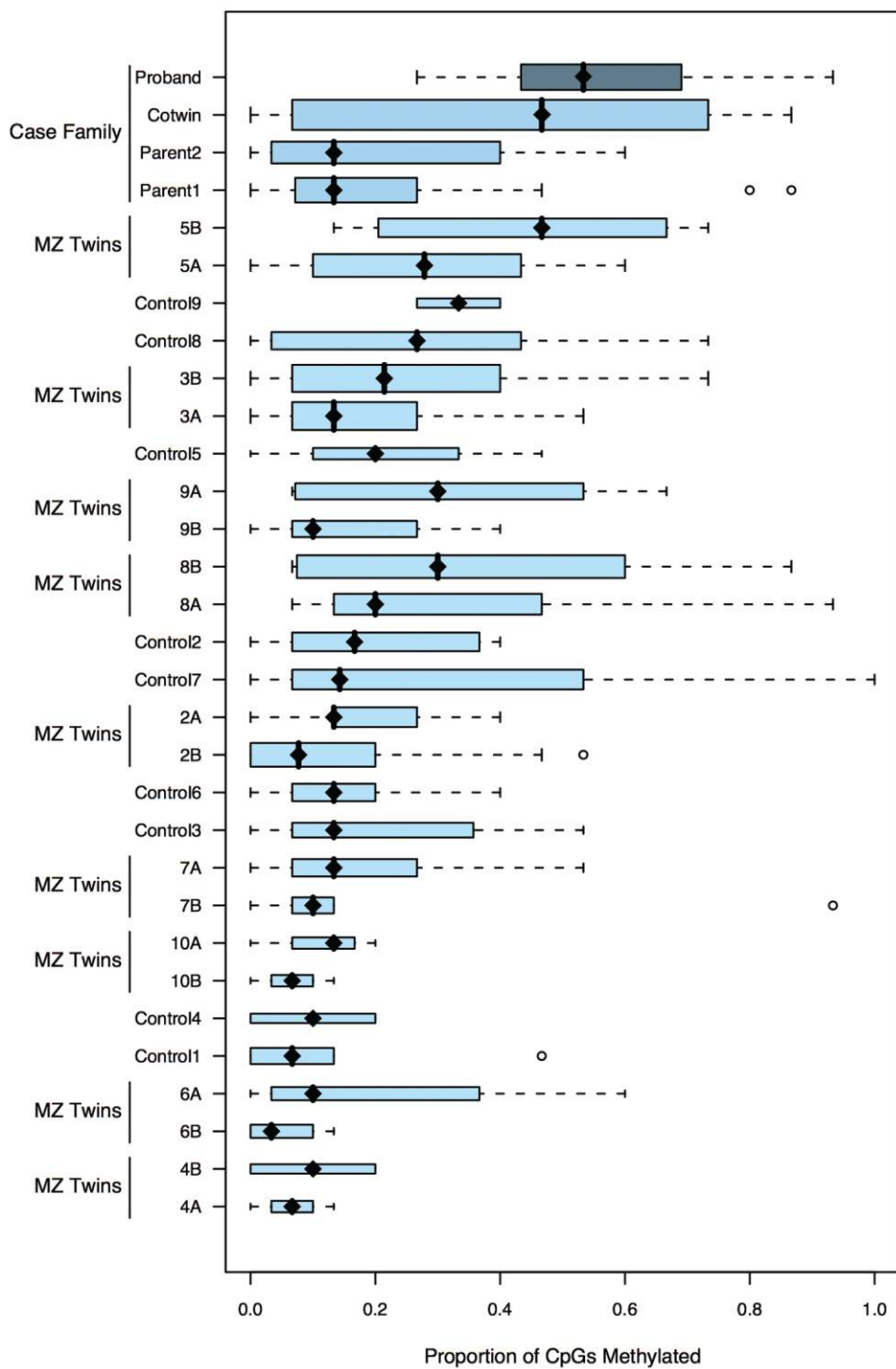
*AXIN1* is positioned near the telomere of human chromosome 16 and has a CpG island at the 5' end (the putative promoter).<sup>15</sup> This CpG island surrounds exon 1 (Ensembl Build 35 [accession numbers 341973-343464]). In intron 2, there is a solitary long terminal repeat (LTR) that exhibits enhancer activity.<sup>16</sup> We used bisulfite sequencing<sup>17</sup> to examine the pattern of cytosine methylation at three distinct regions of *AXIN1*: the 5' CpG island, intron 1, and intron 2-LTR (for PCR primers and conditions, see table A1). The *AXIN1* intron 1 (8 CpGs) and intron 2-LTR (17 CpGs) regions were found to be hypermethylated in PBMC DNA from all individuals tested, with no significant difference between the affected MZ twin and controls. The average percentages of methylated CpGs were, for intron

1, 98% ( $n = 11$ ) for the affected twin and 100% ( $n = 7$ ) for the unaffected twin and, for intron 2-LTR, 80% ( $n = 31$ ) for the affected twin and 88% ( $n = 29$ ) for the unaffected twin. Methylation status of the *AXIN1* 5' CpG island (15 CpGs) was determined for the proband family and for two age-matched controls (fig. 1). This region, in contrast to most of the unmethylated CpG islands in the human genome, is methylated to varying degrees both within and among individuals. The precise pattern of methylation varies considerably from clone to clone in all individuals, including controls. The proportion of the 15 CpGs that are methylated also varies considerably from clone to clone in all individuals (fig. 2). Although whole blood contains a mix of nucleated cell types, the majority are neutrophils, and the complexity of the clonal patterns cannot be explained by this mix alone.

We modeled methylation at 15 CpG sites in twins and controls, using a binomial generalized linear mixed model, as implemented in the *lme4* package<sup>18</sup> in the R statistical programming language.<sup>19</sup> This approach allows the appropriate modeling of within-family and within-individual correlation in the binary (absent or present) methylation status across the different CpG sites. The multilevel random effects were at the level of the family (19 families), individual (4 individuals in the proband family [twins and their parents], 2 in each of the other 9 MZ pairs, and 9 single individuals, for a total of 31 individuals), and clone (3–17 distinct clones per individual, since, in the case of duplicate methylation signatures from the same PCR, one was discarded in case it represented clonal bias; 30 of 311 clones were so discarded). The outcome of the analysis is essentially the same with or without this filtering process. To reduce the impact of any bias, multiple PCRs were undertaken for each sample. Disease status was treated as a fixed effect and its significance was assessed using a likelihood-ratio test. Because of the presence of a SNP within the amplified region of the proband pair, we were able to see that both parental alleles were represented in the data set and that they each showed a range of methylation states (see fig. 1A and 1B).

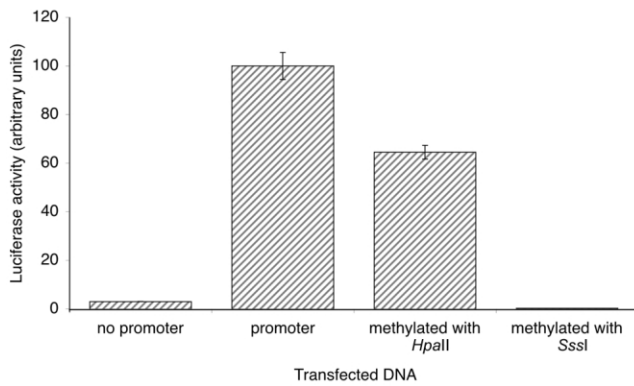
The total level of methylation in the affected twin was significantly higher than that in the unaffected twin ( $P < .0001$ ). The unaffected twin was also significantly more methylated than were controls ( $P = .02$ ). The methylation levels of MZ twins differ from one another but are more similar to each other than they are to unrelated individuals. We estimated the intraclass correlation for total methylation at all 15 sites to be 0.76 ( $P = .02$ ) for all 10 MZ pairs. Excluding the proband pair, the correlation was

**Figure 1.** *AXIN1* CpG island methylation in the MZ twin pair (A, unaffected; B, affected) discordant for caudal duplication anomaly, their parents (D), and age- and sex-matched controls (C). An open circle represents an unmethylated CpG, a filled circle represents a methylated CpG, and each row of circles represents the methylation state of all 15 CpGs on a single allele. The square represents a SNP (G→T [dbSNP rs12928797]) commonly found in the samples analyzed. Overall percentage of methylation was calculated by determining the proportion of methylated CpGs relative to all CpGs. A detailed analysis of the methylation state at each CpG site is also shown (E).



**Figure 2.** Box-and-whisker plot of proportion-methylated CpG sites in the *AXIN1* promoter per clone for each individual. Individuals are ordered by familial mean methylation level, with the index family at the top. Length of bar is interquartile range, and bar width is proportional to the number of clones analyzed for that individual. Solid diamonds are medians, and dashed lines extend to the observations  $1.5 \times$  the interquartile range from the median, with open circles representing more-extreme values.





**Figure 3.** The degree of methylation of the *AXIN1* promoter correlates with its activity in HEK 293 cells. A fragment of the *AXIN1* locus, corresponding to nucleotides  $-416$  to  $+114$  relative to the start point of transcription (as determined by Evidence Viewer), was amplified by PCR from human genomic DNA (Promega). For a more detailed description of the methods used, see the main text. A similar trend was also seen in HeLa cells (data not shown). Each data point is the average of six replicates. Error bars represent two SDs.

still 0.52. The MZ twin correlation in methylation status at each of the 15 sites varied between 0.01 and 0.86, but, with only 10 pairs available, this is still in keeping with homogeneity, so we cannot say whether there is differential genetic control of methylation between sites. The results suggest that the relatively high methylation levels that we observe at the *AXIN1* CpG island in the affected and unaffected MZ twins are unlikely to be associated with twinning itself, since the MZ twin controls do not have significantly higher levels of methylation than the singleton controls ( $P = .73$ ) (fig. 2).

For eight of these individuals (the proband pair, their parents, and four other unaffected singles), we had buccal DNA in addition to PBMC DNA. Buccal epithelia, unlike PBMC, is ectodermal in origin. Methylation of CpG sites in buccal DNAs was absent from all sites in all individuals, including the proband pair and their parents (data not shown), so the variable hypermethylation at the *AXIN1* 5' CpG island is tissue specific.

To confirm that this CpG island functions as a promoter, we cloned a fragment corresponding to nucleotides  $-416$  to  $+114$  relative to the start site of transcription, upstream of a luciferase reporter gene, and we performed transient transfections into the human cell line HEK293. The fragment did function as a promoter, and in vitro methylation of the construct decreased expression; partial methylation (16% of CpGs) resulted in a reduction of expression, and complete methylation resulted in no expression (see fig. 3). Similar results were obtained after transfection into HeLa cells (data not shown).

The primers used to generate this insert were as follows. The upstream primer contained a *BglIII* site followed by

the *AXIN1* sequence, CCAGTCTAGATCTCCACGCTCC-TCACTTTATCCT. The downstream primer contained a *HindIII* site followed by the *AXIN1* sequence, CCAGTCAA-GCTTGAGCCCGGCCCTACTCAC. The insert was cloned into pGL3-Basic (Promega). The resulting construct was methylated in vitro by either *HpaII* methylase (New England Biolabs) or *SssI* methylase (New England Biolabs). Methylation was verified by digestion with the methylation-sensitive restriction enzyme, *HpaII* (New England Biolabs). pRL-Luc was cotransfected, to control for transfection efficiency. Methylation of 16% of the CpG dinucleotides in the *AXIN1* insert by *HpaII* correlated with a 36% reduction in luciferase activity, whereas methylation of 100% of the CpG dinucleotides by *SssI* was associated with a complete silencing of the luciferase gene.

Since the proband pair are female, it is possible that unequal skewing of X inactivation resulting in expression differences in X-linked genes and in any downstream autosomal genes could underlie the phenotypic discordance in the proband twin pair. Analysis of methylation, by use of the human androgen gene assay,<sup>20</sup> showed some evidence for skewing in both the proband and her cotwin in PBMC DNA, but the pattern was identical in both (fig. 4). This finding is consistent with a previous study<sup>21</sup> that showed that most monozygotic MZ twins display highly similar X-inactivation patterns, and it rules out skewed X inactivation as an explanation for the discordance in this instance.

We were curious to discover whether other neighboring CpG promoters were also methylated in PBMC in the proband. The  $\alpha$ -globin locus (*HBA1* [MIM 141800] and *HBA2* [MIM 141850]) lies 170 kb downstream of the *AXIN1* gene. Bisulfite analysis of 16 CpGs in the *HBA1* CpG island promoter showed overall hypomethylation (0%–17%) in all individuals tested, including the proband, the unaffected cotwin, the two parents, and a pair of control MZ twins (data not shown). These results are consistent with previous reports that the  $\alpha$ -globin promoters are relatively unmethylated in PBMCs of normal individuals<sup>22</sup> and suggest that the hypermethylation observed in the proband is specific to the *AXIN1* CpG island.

It is commonly held that, in normal cells, CpG islands are unmethylated, except when they are located on the inactive X chromosome of females or in the differentially methylated regions (DMRs) of imprinted genes. Hypermethylation of normally unmethylated CpG islands is associated with transcriptional silencing. Our analysis of the *AXIN1* CpG island in MZ twins discordant for caudal du-

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

**Figure 4.** Quantification of X-chromosome inactivation in PBMCs. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

plication anomaly identified the affected twin as the individual with the highest methylation state, which may explain the disease phenotype. However, some degree of *AXIN1* CpG island methylation is observed even in unaffected individuals. *AXIN1* is not an X-linked gene and does not show the characteristic allele-specific methylation patterns of an imprinted gene. Presumably, it represents a recently identified class of variably methylated CpG islands in the human genome, which are not DMRs of imprinted genes and are not on the inactive X chromosome.<sup>23</sup> It is interesting that these elements have been shown to map to telomere-proximal positions on several different chromosomes,<sup>23</sup> since *AXIN1* lies at the telomere of the short arm of human chromosome 16.<sup>15</sup>

The variability in methylation at the *AXIN1* CpG island, both within an individual and between individuals, is interesting. Yamada and colleagues used methylation-sensitive restriction enzyme digestion to identify 8 CpG islands (of 149 analyzed on chromosome 21q) that exhibited so-called incomplete methylation, where several CpGs on the same allele are differentially methylated,<sup>9</sup> similar to those reported in this article. All eight were located at promoter or 5'-UTR gene regions, but differences in methylation between individuals were not addressed.<sup>9</sup> Interindividual variation in methylation state, though not necessarily at CpG islands, was reported at the major histocompatibility complex region of human chromosome 6 and, similar to our results, this variation was not observed in every tissue.<sup>24</sup> The consistent hypomethylation of the 5' CpG island of *AXIN1* in buccal tissue suggests that the methylation occurred after differentiation of the three germ layers, since buccal tissue is ectodermal in origin, whereas blood is mesodermal. It is worth noting that mesoderm gives rise to the notochord, which defines the body's axis and eventually becomes the vertebral column. Ideally, we would examine methylation in the vertebral column, but results from a tissue (blood) derived from the same embryonic tissue layer are more likely to reflect the relevant epigenetic state than results from a tissue (buccal epithelium) that is not.

To the best of our knowledge, this study of human *AXIN1* is the first detailed analysis, by bisulfite sequencing,

of a CpG island promoter that exhibits variable methylation both within a tissue and among individuals and, in particular, between genetically identical individuals. Such alleles are referred to as "metastable epialleles" in mice and plants.<sup>3</sup> On the basis of what we have learned about their behavior in other organisms,<sup>3,25-27</sup> it is likely that they will be sensitive both to genetic background and to environment, properties we should expect in agents of sporadic and complex disease. Although, in this instance, a candidate-gene approach was appropriate, in many cases where no obvious candidates are available, newly developed genomewide approaches<sup>13,28</sup> are likely to be fruitful.

Although we favor the hypothesis that the hypermethylation at *AXIN1* in the proband is a purely epigenetic event, a genetic rearrangement in *cis* is almost impossible to rule out, since we know that such events can affect the epigenetic state over long distances.<sup>29</sup> It is also possible that blood supply differences in utero or other postzygotic events<sup>30,31</sup> could be underlying causes of both the phenotypic discordance and the increased methylation levels. We are aware of this and other limitations of our study but believe nevertheless that MZ twins provide the best opportunity to understand the relationship between epigenotype and phenotype in humans, and we hope that our findings make a useful contribution to an inherently difficult field.

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## Appendix A

**Table A1. Primers and Conditions Used for Amplification of *AXIN1* and *HBA1* Promoter Regions**

Region and Primer Name	PCR Round	Primer Sequence
<i>AXIN1</i> 5' CpG island:		
ACGL1	1 and 2	5'-GGAGGTTTTGGTTTTTAGAGAGYGGAG-3'
ACGR1	1	5'-AAACCCCTAACCATCCCTACCTACCRACC-3'
ACGR1.5	2	5'-TCCTAAAAACCTACTCCCTCAC-3'
<i>HBA15'</i> CpG island:		
HBAL1	1 and 2	5'-TTTTATAGTTTAGAGAGAATTTATTATGG-3'
HBAR1	1	5'-TAACCCCTAACCTAAACAAAACC-3'
HBAR2	2	5'-AAAAAAAACAAAACATCCTAC-3'
<i>AXIN1</i> intron 1:		
AxIL1	1	5'-TGTTTATAATTTTAGTTATTTGGGAAGGT-3'
AxIR1	1	5'-ACCCCTTATTTTACTCACACTTCTATT-3'
AxIL2	2	5'-ATAATTTTAGTTATTTGGGAAGGTTGAG-3'
AxIR2	2	5'-AACTTACTTAAATCCACAACCCTATT-3'
<i>AXIN1</i> intron 2-LTR:		
AxLTRL1	1	5'-GGATAAATATAGAAAAGGGTTAGGAATG-3'
AxLTRR1	1 and 2	5'-ATAAACTAAAAACTCCTCAAATACCAC-3'
AxLTRL2	2	5'-TTTTAAATTAAGTATGTGAAATTATTA-3'

NOTE.—A 263-bp fragment of the *AXIN1* 5' CpG island and a 256-bp fragment of the *HBA1* promoter regions were amplified using semi-nested bisulphite PCR. The PCR protocol involved 2 min at 94°C followed by 36 cycles for 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, with a final extension of 6 min at 72°C. The PCR contained BSA (4 mg/mL) and, for the *AXIN1* 5' CpG island region, Polymate additive (Bioline) as well. The resulting PCR fragments were subcloned into the p-GEM-T Easy Vector (Promega) and were sequenced.

### Web Resources

Accession numbers and URLs for data presented herein are as follows:

dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/> (for *rs12928797*)  
 Ensembl Build 35, <http://www.ensembl.org> (accession numbers 341973-343464)  
 Evidence Viewer, <http://www.ncbi.nlm.nih.gov/>  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for caudal duplication anomaly, *AXIN1*, *HBA1*, and *HBA2*)

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