Genetic factors are major determinants of phenotypic variability in a mouse model of the DiGeorge/*del22q11* syndromes

Ilaria Taddei, Masae Morishima, Tuong Huynh, and Elizabeth A. Lindsay*

Department of Pediatrics (Cardiology), Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030

Edited by David E. Housman, Massachusetts Institute of Technology, Cambridge, MA, and approved July 23, 2001 (received for review March 14, 2001)

The *del22q11* syndrome is associated with a highly variable phenotype despite the uniformity of the chromosomal deletion that causes the disease in most patients. *Df1*/+ mice, which model *del22q11*, present with reduced penetrance of cardiovascular defects similar to those seen in deleted patients but not with other *del22q11*-like findings. The reduced penetrance of cardiovascular defects is caused by the ability of mutant embryos to recover from a fourth pharyngeal arch artery growth abnormality that is fully penetrant in early embryos. Here we show that genetic background has a major effect on penetrance of cardiovascular defects by affecting this embryonic recovery process. This effect could not be explained by allelic variation at the haploid locus, and it is likely to be caused by genetic modifiers elsewhere in the genome. We also show that genetic factors control extension of the *Df1*/+ phenotype to include thymic and parathyroid anomalies, establishing the *Df1* mouse as a model for the genetic analysis of three major features of human *del22q11* syndrome. We found that in *Df1*/+ mice, as in human patients, expression of the heart and thymic phenotypes are essentially independent from each other, suggesting that they may be controlled by different genetic modifiers. These data provide a framework for our understanding of phenotypic variability in patients with *del22q11* syndrome and the tools for its genetic dissection.

The *del22q11* syndrome, which includes DiGeorge syndrome (DGS), velocardiofacial syndrome, and conotruncal anomaly face is the most common microdeletion syndrome, occurring in ≈1:4,000 live births (†, 1). The *del22q11* syndrome is caused by heterozygous deletions in the chromosome region 22q11.2. It has been proposed that deletions may result from aberrant homologous recombination between low copy repeat sequences that flank the deleted region (2, 3). The majority of patients (>85%) has a common ≈3-megabase (Mb) deletion encompassing ≈30 genes, and ≈8% has an ≈1.5-Mb nested deletion encompassing 24 genes, and there are rare cases with alternative deletions and translocations (3–5). *del22q11* syndrome can be associated with a broad phenotype, but the most characteristic features are congenital heart disease, especially defects of the aortic arch and cardiac outflow tract and thymic and parathyroid aplasia or hypoplasia, which may cause T cell immune deficiencies and hypocalcemia, respectively. Other common findings are facial anomalies, learning difficulties or mild mental retardation, and renal and skeletal anomalies.

Despite the uniformity of the common deletion, the *del22q11* syndrome phenotype is characterized by reduced penetrance of the various phenotypic components and variable expressivity (1, 6, 7). In particular, no consistent difference is seen in the phenotype associated with the 3- or 1.5-Mb deletions (8). The basis of the phenotypic variability is not understood. To address the potential role of genetic factors in determining the *del22q11* phenotype, we have used a mouse model of *del22q11* syndrome that we developed recently by using chromosome engineering (9). The mouse deletion (*Df1*) spans ≈1 Mb and encompasses 18 mouse homologs of genes deleted in *del22q11* syndrome patients. All the genes within *Df1* are represented in the 1.5-Mb deletion (and therefore also in the 3-Mb deletion), although there are some changes in gene order caused by ancestral rearrangements (10, 11). Similar to human patients, heterozygously deleted mice (*Df1*/+) show reduced penetrance of *del22q11* syndrome-like heart defects, namely interrupted aortic arch type-B, right aortic arch, aberrant origin of the right subclavian artery, overriding aorta, pulmonary stenosis, and ventricular septal defects. We have demonstrated that this cardiovascular phenotype is caused by gene haploinsufficiency (9). However, on the mixed C57BL/6;129SvEv genetic background (hereafter referred to as mixed) used to determine the reported phenotype, *Df1*/+ mice do not manifest other features of the *del22q11* syndrome phenotype (9). We hypothesized that this may be because *Df1*, which is smaller than *del22q11*, does not include the relevant genes or because phenotypic expression of extracardiac defects depends on genetic modifiers located elsewhere. The results presented here strongly support the latter hypothesis and indicate that genetic factors are major (but not sole) determinants of penetrance of the *del22q11* syndrome-like phenotype in mice.

Methods

Mouse Breeding and Genotyping. The *Df1* deletion was generated in embryonic stem cells derived from an inbred 129SvEvBrd mouse strain, hereafter referred to as 129SvEv. To obtain the deletion in a pure 129SvEv background, we bred chimeric mice with inbred 129SvEv females. In this breeding, pups carrying the *Df1* deletion will have both paternal (from embryonic stem cells) and maternal chromosome complements of the 129SvEv origin. To obtain the *Df1* deletion on a congenic C57BL/$6^{c-/c-}$ background (hereafter referred to as C57BL/6), we back-crossed *Df1*/+ mice for nine generations with C57BL/$6^{c-/c-}$ inbred mice. To generate *Df1*/+ embryos that are identical genetically and have a normal (nondeleted) chromosome 16 of C57BL/6 origin, we crossed inbred 129SvEv *Df1*/+ mice with wild-type C57BL/6 mice. Because there is no evidence of imprinting in the human disease and we see no difference in the *Df1*/+ phenotype whether the deletion is inherited maternally or paternally, we chose to use *Df1*/+ males and wild-type females for these experiments. The *Df1* progeny of this mating (hereafter referred to as F_1 hybrid) are different than those of matings between mice of the mixed genetic background reported previously (9), in which the haploid region of chromosome 16 could be of C57BL/6 or 129SvEv origin. In the latter case, the genetic makeup of the embryos analyzed was of three types: 50:50

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Mb, megabase(s); E, embryonic day; PAA, pharyngeal arch artery.

*To whom reprint requests should be addressed at: Department of Pediatrics (Cardiology), Baylor College of Medicine, 325D, 1 Baylor Plaza, Houston, TX 77030. E-mail: elindsay@bcm.tmc.edu.

†Wilson, D. I., Cross, I. E., Wren, C., Scambler, P. J., Burn, J. & Goodship, J. (1994) *Am. J. Hum. Genet.* **55,** Suppl., A975 (abstr.).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

 www.pnas.org/cgi/doi/10.1073/pnas.201127298

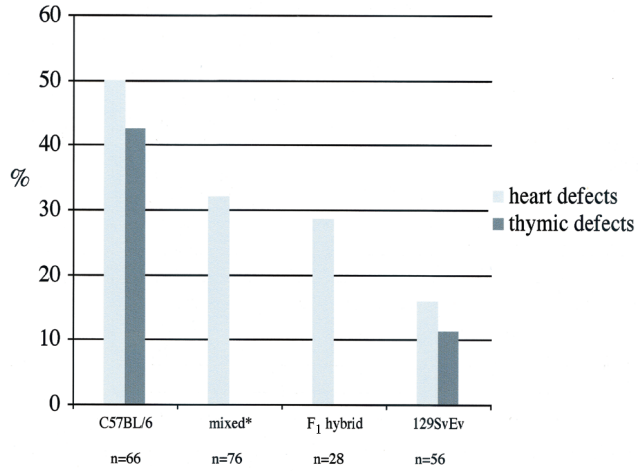

Fig. 1. Penetrance of cardiovascular and thymic defects in term *Df1*/+ embryos in four different genetic backgrounds. The penetrance of both cardiovascular and thymic defects in congenic C57BL/6 embryos was higher than in the other backgrounds tested and is similar to the penetrance of these defects in patients with *del22q11* syndrome. F_1 hybrid mice, which are the progeny of 129SvEv *Df1*/+ males and wild-type C57BL/6 females, are identical genetically, and the normal (nondeleted) chromosome 16 is C57BL/6-derived, whereas in mixed mice the haploid region of chromosome 16 may be C57BL/6- or 129SvEv-derived. *, These data are taken from Lindsay and Baldini (13) and are shown here for comparison.

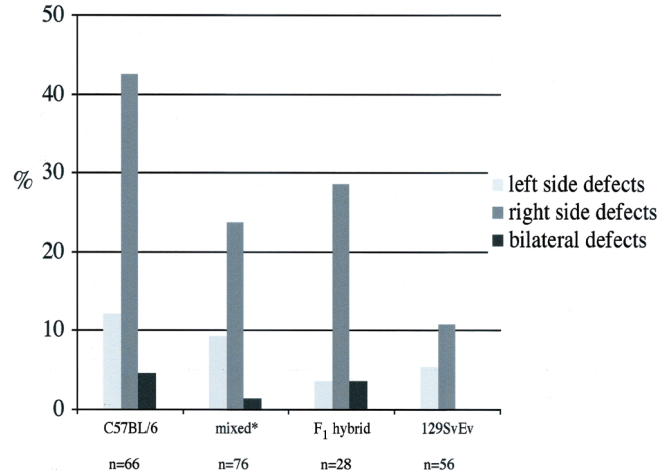

Fig. 2. Percentage of left, right, and bilateral cardiovascular defects in term *Df1*/+ embryos in four different genetic backgrounds. Right-sided defects predominated in all genetic backgrounds, but the proportion of left, right, and bilateral defects was similar in all backgrounds.

129SvEv/C57BL/6, 75% C57BL/6, 25% 129SvEv or 75% 129SvEv, and 25% C57BL/6. Genotyping was carried out on DNA extracted from yolk sacs. In all the strains tested the deletion was transmitted in a Mendelian fashion at E18.5.

Ink Injection, Histology, and *in Situ* Hybridization. For the analysis of pharyngeal arch artery anatomy, E10.5 embryos were injected intracardially with India ink and fixed overnight in a mixture of 95% ethanol, 1% chloroform, and 1% acetic acid followed by clearing in 1:1 methyl salicylate/benzyl benzoate. For histology and RNA *in situ* hybridization, embryos were fixed overnight in PBS containing 4% paraformaldehyde, embedded in paraffin wax, and cut into 10-μm sections. The parathyroid glands were identified on histological sections counterstained with periodic acid Schiff and hematoxylin. RNA *in situ* hybridization was performed in accordance with a published protocol (12).

Results

The Penetrance of Cardiovascular Defects Varies Widely in Different Genetic Backgrounds. For each genetic background, we bred *Df1*/+ males with wild-type females and collected and dissected embryos at E18.5 to analyze the great arteries and the internal anatomy of the heart. In 129SvEv *Df1*/+ embryos, we observed a lower penetrance of cardiovascular abnormalities (16.1%) than the ≈32% penetrance that we had observed in *Df1*/+ embryos on the mixed genetic background (Fig. 1). In contrast, in C57BL/6 *Df1*/+ embryos, we found a much higher penetrance (50%) of cardiovascular defects. We did not observe differences among the inbred, congenic, or mixed strains with regard to the types of great artery defects seen, and the proportion of left-sided (interrupted aortic arch type-B, right aortic arch) vs. right-sided (aberrant origin of the right subclavian artery) defects and of bilateral defects was similar in all strains (Fig. 2). In view of the high penetrance of aortic arch defects in congenic C57BL/6 *Df1*/+ embryos, we analyzed the internal heart anatomy of these embryos for evidence of increased penetrance or severity of intracardiac defects, but results were not different than those reported in *Df1*/+ embryos on the original mixed genetic background (9).

The embryological basis for most of the *Df1*/+ cardiovascular defects is abnormal development of the fourth pharyngeal arch arteries (PAAs), which can be identified by intracardiac ink injection (9). We have reported recently that at E10.5, the fourth PAA phenotype is fully penetrant in *Df1*/+ embryos in the mixed genetic background, after which ≈70% of defective arteries recovers and develops normally (13). The differences in penetrance of the cardiovascular phenotype observed in the inbred and congenic strains at term could be reflecting earlier embryological events. We therefore analyzed *Df1*/+ embryos on the129SvEv (n = 20) and C57BL/6 (n = 30) backgrounds at E10.5 by intracardiac ink injection and found that the fourth PAA phenotype was fully penetrant irrespective of genetic background. Furthermore, in accordance with our observations of term embryos on these genetic backgrounds, there were no strain-related differences in the proportion of left vs. right sided abnormalities or of bilateral defects (data not shown).

Allelic Variation Within *Df1* Does Not Account for Increased Penetrance of the Cardiovascular Phenotype in the C57BL/6 Background. To test whether the high penetrance of cardiovascular defects in C57BL/6 *Df1*/+ embryos is caused by the presence of a hypomorphic allele of the haploinsufficient gene on the normal chromosome 16 in this strain, we generated embryos in which the *Df1*/+ progeny (F_1 hybrid) have the haploid region of C57BL/6 origin (see *Methods*). If a hypomorphic allele was solely responsible for the high penetrance, F_1 hybrid embryos would have a similarly penetrant phenotype as congenic C57BL/6 mice. However, we found that this was not the case, and the penetrance of cardiovascular defects in the F_1 hybrid *Df1*/+ embryos (28.6%) was similar to that of a series of 76 *Df1*/+ embryos of mixed

Table 1. Heart and thymic defects occur independently of each other

Df1/+ embryos with heart and/or thymic anomalies	C57BL/6 (n = 25)	129SvEv (n = 10)
Thymus + heart	19 (76%)	2 (20%)
Thymus only	2 (8%)	3 (30%)
Heart only	4 (16%)	5 (50%)

GENETICS

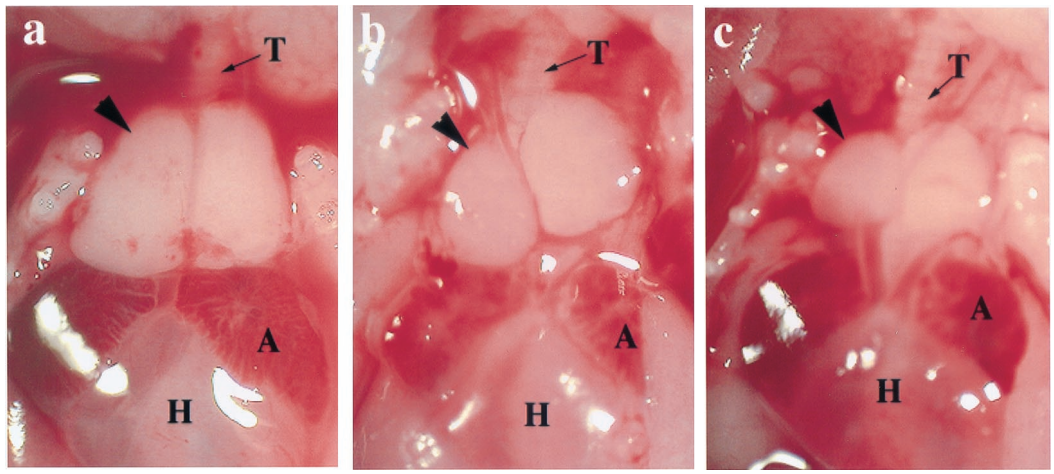

Fig. 3. Thymic anomalies in congenic C57BL/6 *Df1*/+ embryos. Frontal views of the mediastinum of E18.5 wild-type (*a*) and *Df1*/+ (*b* and *c*) embryos. The lobes of the thymus (arrowheads) show variable degrees of hypoplasia and asymmetry in *Df1*/+ embryos compared with wild-type litter mates. H, heart; A, auricle; T, trachea.

genetic background (Fig. 1) that we reported recently (13), suggesting that allelic variation within the haploid segment is not the sole determinant of penetrance of cardiovascular defects in the C57BL/6 background.

Thymic and Parathyroid Anomalies Appear in Inbred and Congenic Strains. Analysis of E18.5 embryos revealed thymic anomalies in *Df1*/+ embryos in the congenic C57BL/6 background (42.5%) and in the inbred 129SvE background (11.3%) (Table 1). Thymic defects were not found in F_1 hybrid *Df1*/+ embryos or in the original mixed genetic background (9). Normally, at E18.5 the thymus is descended completely into the mediastinum and is composed of two essentially symmetrical lobes with a commessure between the two lobes at the midline (Fig. 3*a*). The morphological anomalies found in C57BL/6 and 129SvEv *Df1*/+ embryos included lobe asymmetry, hypoplasia of one or both lobes (the right lobe being more commonly affected than the left), and incomplete descent of the thymus into the mediastinum (Fig. 3, *b* and *c*). Thymic anomalies and cardiovascular defects did not always occur together (Table 1).

In view of the high penetrance of cardiovascular defects and thymic anomalies in congenic C57BL/6 embryos, we selected *Df1*/+ embryos of this strain to analyze parathyroid gland development. Analysis of histological sections of E18.5 embryos revealed that in all 21 *Df1*/+ embryos examined, one or both parathyroids were located in an anomalous position medial to the thyroid, between the thyroid and the trachea (Fig. 4*a*), whereas, in 16 of 18 wild-type embryos examined, the parathyroids were located lateral to the thyroid (Fig. 4*b*). To determine whether this position anomaly could be associated with developmental anomaly of the glands, we analyzed E12.5 *Df1*/+ embryos for early parathyroid development defects by RNA *in situ* hybridization with the parathyroid-specific marker *Pth*. We found that the area (but not the intensity) of the *Pth* signal was reduced by ≈75% in *Df1*/+ embryos compared with wild-type embryos (Fig. 5), suggesting that in *Df1*/+ embryos early development of the parathyroids is abnormal or delayed, and the number of *Pth*-expressing cells is reduced.

Discussion

The clinical presentation of human *del22q11* syndrome can vary widely amongst patients in the same sibship (6, 7), and at least for the cardiovascular phenotype, even amongst monozygotic twins (14–17). This picture would suggest that expression of the phenotype is controlled largely by nongenetic factors. However, because humans are highly outbred and the monozygotic twin cases reported are very few at this time, it is not possible to assess the contribution of genetic factors to phenotypic variability in humans. The availability of a mouse model circumvents the typical difficulties of testing human populations. The data presented here clearly show that the

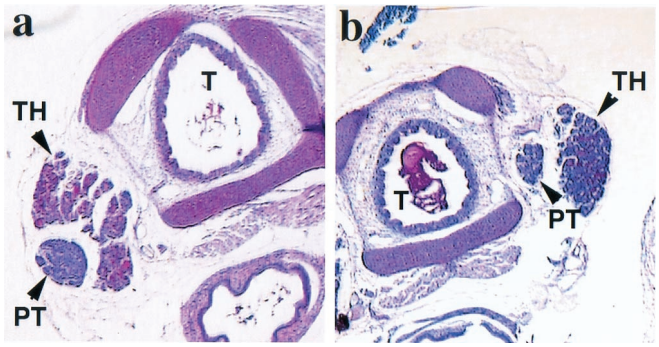

Fig. 4. Parathyroid gland anomalies in congenic C57BL/6 *Df1*/+ embryos. Transverse sections through the thyroid and parathyroid glands of E18.5 embryos showing the normal position of the parathyroid gland in a wild-type embryo (*a*) and the anomalous position in a *Df1*/+ embryo (*b*) are shown. TH, thyroid; PT, parathyroid; T, trachea.

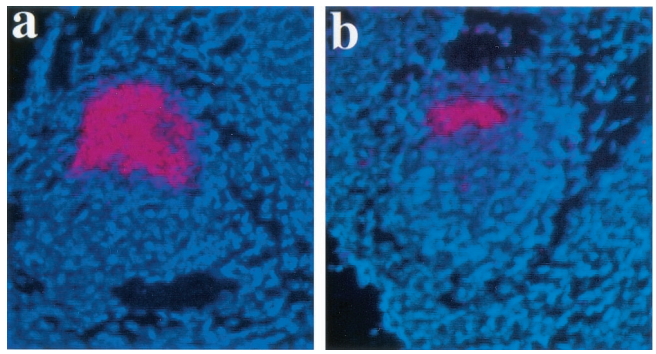

Fig. 5. *Pth* expression in congenic C57BL/6 *Df1*/+ embryos. RNA *in situ* hybridization of the parathyroid-specific marker *Pth* to sagittal sections of E12.5 wild-type (*a*) and *Df1*/+ (*b*) embryosis shown. The area of *Pth* expression in *Df1*/+ embryos is reduced to ≈25% that of wild-type levels.

penetrance of congenital heart disease is controlled to a great extent by genetic factors. However, the incomplete penetrance observed in the inbred and congenic strains, notwithstanding the genetic homogeneity of these animals, suggests that stochastic factors are also determinants.

We have shown previously that the penetrance of cardiovascular defects in *Df1/+* embryos at term is determined by the efficiency of a "recovery" process in the development of the fourth PAAs (13). Remarkably, although the penetrance of cardiovascular defects at term is dramatically different in 129SvEv and C57BL/6 animals, we saw no difference in penetrance of the fourth PAA phenotype at E10.5 in the two genetic backgrounds. These data indicate that genetic factors affect the recovery process rather than the primary embryological defect. This is consistent with the observation that the increased penetrance of defects in C57BL/6 animals is not associated with increased severity of the phenotype.

Both the human and mouse phenotypes are caused by gene haploinsufficiency; therefore, allelic variation at the haploid locus may be the genetic basis of phenotypic variability. A "weak" (hypomorphic) allele of the haploinsufficient gene may result in low levels of gene expression, normal expression of an altered gene product, or both. Although our data do not formally exclude the existence of a hypomorphic allele in the haploid locus, they clearly indicate that if it exists, it is not the sole determinant of penetrance of the cardiovascular defects. These findings suggest the presence of modifier genes elsewhere in the genome.

The strain-dependent expression of the thymic phenotype and its incomplete penetrance in the inbred and congenic mutants are reminiscent of the *Df1/+* cardiovascular phenotype. However, the two phenotypes do not always occur together, which is remarkably similar to the human *del22q11* phenotype, where cardiovascular and thymic abnormalities can occur independently (6, 7). These data suggest that the expression of the thymic and cardiovascular phenotypes may be affected by independent genetic modifiers in human and mice. This would not be unexpected, because the thymus and pharyngeal arch arteries are derived from different tissues.

It is unlikely that the thymic and parathyroid anomalies associated with *Df1/+* would have a functional relevance, because the thymic hypoplasia is relatively mild, even in the most severely affected embryo (Fig. 3*c*), and the parathyroids are of normal size, although anomalously positioned. Nevertheless, the anomalies are biologically significant, because they reveal that gene haploinsufficiency in the mouse can model the key developmental defects associated with *del22q11*, provided that it is examined in the appropriate genetic background. This should allow us to establish accurately the genetic mechanisms of phenotypic variability and the molecular pathogenesis. We have established recently that *Tbx1* haploinsufficiency is necessary and sufficient to produce the cardiovascular phenotype in *Df1/+* animals (18). Others have also reported similar results (19, 20). *Tbx1*, which is a T-box gene family member and a putative transcription factor, is required in mouse for the development of the distal pharyngeal arches and pouches (18, 19) from which the thymus and parathyroid glands are derived. Therefore, although our *Tbx1* mutant allele is not yet available in the 129SvEv and C57BL/6 inbred backgrounds, it is reasonable to predict that haploinsufficiency of this gene could cause the thymic and parathyroid anomalies described here. At this point, we do not know the genes targeted by the transcription factor *Tbx1*, but it is conceivable that different target genes will contribute to the development of pharyngeal arch arteries, thymus, and parathyroids. This would provide a molecular framework within which the variability of the phenotype and its genetic control could be interpreted.

In conclusion, the data presented here demonstrate for the first time a genetic control over phenotypic variability of the *del22q11* syndrome-associated phenotype and extend the validity of the haploinsufficiency mouse model for the genetic analysis of this complex syndrome. Our results provide a framework for the identification of the genetic factors controlling phenotypic variability. We hypothesize that these genetic factors may be important components of the genetic network responsible for the development of different derivatives of the pharyngeal arches and pouches.

We thank Dr. Antonio Baldini for critical reading of the manuscript. This work was funded by American Heart Association (Texas Affiliate) Grant 0060099Y (to E.A.L.).

1. Scambler, P. J. (2000) *Hum. Mol. Genet.* **9,** 2421–2426.
2. Edelmann, L., Pandita, R. K. & Morrow, B. E. (1999) *Am. J. Hum. Genet.* **64,** 1076–1086.
3. Shaikh, T. H., Kurahashi, H., Saitta, S. C., O'Hare, A. M., Hu, P., Roe, B. A., Driscoll, D. A., McDonald-McGinn, D. M., Zackai, E. H., Budarf, M. L. & Emanuel, B. S. (2000) *Hum. Mol. Genet.* **9,** 489–501.
4. Lindsay, E. A., Goldberg, R., Jurecic, V., Morrow, B., Carlson, C., Kucherlapati, R. S., Shprintzen, R. J. & Baldini, A. (1995) *Am. J. Med. Genet.* **57,** 514–522.
5. Edelmann, L., Pandita, R. K., Spiteri, E., Funke, B., Goldberg, R., Palanisamy, N., Chaganti, R. S., Magenis, E., Shprintzen, R. J. & Morrow, B. E. (1999) *Hum. Mol. Genet.* **8,** 1157–1167.
6. Lindsay, E. A., Greenberg, F., Shaffer, L. G., Shapira, S. K., Scambler, P. J. & Baldini, A. (1995) *Am. J. Med. Genet.* **56,** 191–197.
7. Ryan, A. K., Goodship, J. A., Wilson, D. I., Philip, N., Levy, A., Seidel, H., Schuffenhauer, S., Oechsler, H., Belohradsky, B., Prieur, M., *et al.* (1997) *J. Med. Genet.* **34,** 798–804.
8. Carlson, C., Sirotkin, H., Pandita, R., Goldberg, R., McKie, J., Wadey, R., Patanjali, S. R., Weissman, S. M., Anyane-Yeboa, K., Warburton, D., *et al.* (1997) *Am. J. Hum. Genet.* **61,** 620–629.
9. Lindsay, E. A., Botta, A., Jurecic, V., Carattini-Rivera, S., Cheah, Y.-C., Rosenblatt, H. M., Bradley, A. & Baldini, A. (1999) *Nature (London)* **401,** 379–383.
10. Puech, A., Saint-Jore, B., Funke, B., Gilbert, D. J., Sirotkin, H., Copeland, N. G., Jenkins, N. A., Kucherlapati, R., Morrow, B. & Skoultchi, A. I. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14608–146013.
11. Sutherland, H. F., Kim, U. J. & Scambler, P. J. (1998) *Genomics* **52,** 37–43.
12. Albrecht, U., Eichele, G., Helms, J. A. & Lu, H. C. (1997) in *Molecular and Cellular Methods in Developmental Toxicology,* ed. Daston, G. P. (CRC Inc., New York), pp. 23–48.
13. Lindsay, E. A. & Baldini, A. (2001) *Hum. Mol. Genet.* **10,** 997–1002.
14. Goodship, J., Cross, I., Scambler, P. & Burn, J. (1995) *J. Med. Genet.* **32,** 746–748.
15. Yamagishi, H., Ishii, C., Maeda, J., Kojima, Y., Matsuoka, R., Kimura, M., Takao, A., Momma, K. & Matsuo, N. (1998) *Am. J. Med. Genet.* **78,** 319–321.
16. Vincent, M. C., Heitz, F., Tricoire, J., Bourrouillou, G., Kuhlein, E., Rolland, M. & Calvas, P. (1999) *Genet. Couns.* **10,** 43–49.
17. Hillebrand, G., Siebert, R., Simeoni, E. & Santer, R. (2000) *J. Med. Genet.* **37,** E23 (lett.).
18. Lindsay, E. A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H. S., Scambler, P. J., *et al.* (2001) *Nature (London)* **410,** 97–101.
19. Jerome, L. A. & Papaioannou, V. E. (2001) *Nat. Genet.* **27,** 286–291.
20. Merscher, S., Funke, B., Epstein, J. A., Heyer, J., Puech, A., Min Lu, M. M., Xavier, R. J., Demay, M. B., Russell, R. G., Factor, S., *et al.* (2001) *Cell* **104,** 619–629.