DOI: 10.1534/genetics.106.067298

A Genetic Screen for Modifiers of the Delta1-Dependent Notch Signaling Function in the Mouse

Isabel Rubio-Aliaga,* Dian Soewarto,*,[1] Sibylle Wagner,* Matthias Klaften,* Helmut Fuchs,* Svetoslav Kalaydjiev,[†] Dirk H. Busch,[†] Martina Klempt,[‡] Birgit Rathkolb,[‡] Eckhard Wolf,[‡] Koichiro Abe,*,[2] Stefan Zeiser,[§] Gerhard K. H. Przemeck,* Johannes Beckers* and Martin Hrabé de Angelis*,[3]

**Institute of Experimental Genetics, GSF Research Center for Environment and Health, 85764 Neuherberg, Germany,* [†]*Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich, 81675 Munich, Germany,* [‡]*Institute of Molecular Animal Breeding, Gene Center, University of Munich, 81377 Munich, Germany,* [§]*Institute of Biomathematics and Biometry, GSF Research Center for Environment and Health, 85764 Neuherberg, Germany*

Manuscript received October 25, 2006
Accepted for publication December 11, 2006

ABSTRACT

The Notch signaling pathway is an evolutionarily conserved transduction pathway involved in embryonic patterning and regulation of cell fates during development. Recent studies have demonstrated that this pathway is integral to a complex system of interactions, which are also involved in distinct human diseases. Delta1 is one of the known ligands of the Notch receptors. Mice homozygous for a loss-of-function allele of the Delta1 gene *Dll1*$^{lacZ/lacZ}$ die during embryonic development. Here, we present the results of two phenotype-driven modifier screens. Heterozygous *Dll1*^{lacZ} knockout animals were crossed with ENU-mutagenized mice and screened for dysmorphological, clinical chemical, and immunological variants that are dependent on the *Delta1* loss-of-function allele. First, we show that mutagenized heterozygous *Dll1*^{lacZ} offspring have reduced body weight and altered specific clinical chemical parameters, including changes in metabolites and electrolytes relevant for kidney function. In our mutagenesis screen we have successfully generated 35 new mutant lines. Of major interest are 7 mutant lines that exhibit a *Dll1*$^{lacZ/+}$-dependent phenotype. These mutant mouse lines provide excellent *in vivo* tools for studying the role of Notch signaling in kidney and liver function, cholesterol and iron metabolism, cell-fate decisions, and during maturation of T cells in the immune system.

THE Notch signaling pathway is an intercellular signaling mechanism that is highly conserved during evolution. It is involved in the determination of cell fates in different cell types of metazoan development. The phenotype caused by *Notch* gene mutation was first observed almost 90 years ago in *Drosophila melanogaster* as a notched wing (Artavanis-Tsakonas *et al.* 1999). Complete loss of function of the *Notch* gene leads to a lethal phenotype in flies; cells destined to become epidermis switch their fate and give rise to neural tissue. Genes encoding *Notch* orthologs have been cloned and characterized in several organisms. In mammals, four distinct *Notch* genes have been identified, *Notch1–4* (Ellisen *et al.* 1991; Weinmaster *et al.* 1992; Lardelli *et al.* 1994; Uyttendaele *et al.* 1996). The Notch receptors are anchored to the plasma membrane via a single transmembrane domain. Two families of transmembrane ligands interact with the Notch receptors: the Delta and the Jagged/Serrate class of gene products (Kadesch 2004). In mammals, the Delta family encompasses three proteins, Delta1, Delta3, and Delta4 (Bettenhausen *et al.* 1995; Dunwoodie *et al.* 1997; Shutter *et al.* 2000), and the Jagged/Serrate family consists of two ligands, Jagged1 and Jagged2 (Lindsell *et al.* 1995; Shawber *et al.* 1996). The canonical Notch pathway involves two distinct proteolytic processing events that cleave the Notch receptors in response to ligand activation. This eventually leads to the release of the *N*otch *i*ntra*c*ellular *d*omain (NICD) into the cytoplasm (Kadesch 2004). NICD is subsequently translocated to the nucleus, where it interacts with the transcriptional regulator *C*BF, *S*u(H), *L*ag-2 (CSL) (Jarriault *et al.* 1995), converting CSL from a repressor to an activator and leading to the transcription of a variety of target genes, including members of the Hes and HRT/HERP/Hey families of transcriptional repressors. These genes encode basic helix-loop-helix transcriptional factors that downregulate the expression of Notch target genes influencing numerous biological processes (Kadesch 2004; Bianchi *et al.* 2006).

[1]*Present address:* Center for Cardiovascular Research, Hessische Strasse 3-4, 10115 Berlin, Germany.

[2]*Present address:* Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University, Kanagawa 259-1193, Japan.

[3]*Corresponding author:* Institute of Experimental Genetics, GSF Research Center for Environment and Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany. E-mail: hrabe@gsf.de

Loss- or gain-of-function mutations of the receptors or ligands of the Notch signaling pathway have several clinical, physiological, and biological consequences in humans (Harper *et al.* 2003). For example, cancer-like T-cell acute lymphoblastic leukemia [Online Mendelian Inheritance in Man (OMIM) no. 190198] is associated with mutations in *Notch1* (Ellisen *et al.* 1991); cerebral arteriopathy autosomal dominant with subcortical infarcts and leukoencephalopathy (CADASIL; OMIM no.125310), a neurological disease, is associated with mutations in *Notch3* (Joutel *et al.* 1996); Alagille syndrome (OMIM no. 118450), a developmental disease, is associated with mutations in *Jagged1* (Li *et al.* 1997; Oda *et al.* 1997); and spondylocostal dysostosis (OMIM no. 277300), leading to severe congenital malformations of the vertebral column, is associated with mutations in *Delta3* (Bulman *et al.* 2000).

The above-described canonical linear representation of Notch activity is a simplified model as the full pathway is integrated into a complex system of regulatory interactions with pleiotropic functions. For example, recent studies indicate that not only the intracellular domain of the Notch receptor interacts with other proteins, but also the ICD of the ligands Jagged1 and Delta1 (LaVoie and Selkoe 2003; Pfister *et al.* 2003) may initiate transduction cascades in the ligand-expressing cells. After proteolytic processing of the receptor, the released extracellular domain of Notch, moreover, may undergo endocytosis in the ligand-expressing cell (Parks *et al.* 2000) or may interact with proteins such as MAGP-2 to initiate a separate signaling cascade (Miyamoto *et al.* 2006). Moreover, Notch may function independently of CSL proteins (Martinez Arias *et al.* 2002; Bianchi *et al.* 2006) or through interaction with other pathways such as TGFβ signaling (Kluppel and Wrana 2005). Other branches in the canonical signaling pathway have been described and more still remains to be elucidated.

One way to identify either direct or indirect participants in a molecular network or biological process is to perform genetic modifier screens. These already have proven to be highly efficient in the fly and recently also in the mouse (Cormier *et al.* 2000; Carpinelli *et al.* 2004; Curtis 2004; Murcia *et al.* 2004; Speca *et al.* 2006). *N*-**e**thyl-*N*-**n**itroso**u**rea (ENU) mutagenesis has been used as a powerful tool for the establishment of mutant mouse lines that model diseases and for the elucidation of protein function (Hrabe de Angelis *et al.* 2000; Nolan *et al.* 2000). One of the main advantages of ENU mutagenesis is the generation of point mutations that result in functional changes ranging from hypomorphic to full loss of function to hypermorphic alleles. These often provide more functional information and are usually more relevant for human diseases than classical knockouts where the function of a gene is usually completely eliminated (Balling 2001). Therefore, we are running a phenotype-driven dominant modifier screen by mating ENU-mutagenized mice with *Delta1* heterozygous animals to discover novel modifier alleles of the Notch signaling pathway. The *Delta1* knockout mouse line was previously generated by replacing amino acids 2–116 with an in-frame fusion of the *LacZ* gene of *Escherichia coli* (Hrabe de Angelis *et al.* 1997). Delta1 loss of function resulted in developmental lethality before 12.5 days postcoitum. Delta1-deficient embryos showed deficits in epithelialization and compartmentalization of somites and excessive neuronal differentiation. Furthermore, Delta1-deficient embryos showed alterations in cell-fate determination in the developing pancreas (Apelqvist *et al.* 1999) and in the determination of left–right asymmetry in mice (Przemeck *et al.* 2003). Conditional ablation of Delta1 function in lymphoid tissue has revealed its importance for the generation of marginal B cells but not for T cells (Hozumi *et al.* 2004). Selective loss of Delta1 function in the developing ear resulted in an early and excessive development of auditory hair cells (Brooker *et al.* 2006). Recently, it has been demonstrated that Delta1 heterozygous mice show homeotic transformations in the cervical region in vertebral identities with incomplete penetrance (Cordes *et al.* 2004). Here, we report that heterozygous *Delta1* loss-of-function animals show additional variant phenotypes, including alterations in clinical chemical parameters; and these effects are dependent on the genetic background of the animals. Moreover, heterozygous $Dll1^{lacZ}$ animals display a significantly reduced body weight. In our dominant modifier genetic screen we are searching for novel variants with new mutant phenotypes that are dependent on the *Delta1* modified allele. We cover a broad spectrum of phenotypes by analyzing several dysmorphological, clinical chemical, and immunological parameters. At present seven *Delta1*-dependent heritable mutant phenotypes have been identified, all of which are co-inherited with a single $Dll1^{lacZ}$ allele and undetectable in the $Dll1^{+/+}$ background. These novel mouse mutant lines will provide important information about modifiers of Delta1 signaling and the extended Notch pathway.

MATERIALS AND METHODS

Animals: Mice were housed and handled according to the federal animal welfare guidelines and all the animal studies were approved by the state ethics committee. For this study we used C57BL/6J mice provided by Charles River (Wilmington, MA) and mice carrying the $Dll1^{lacZ}$ allele ($Dll1^{tm1Gos}$) (Hrabe de Angelis *et al.* 1997) on an 129X1/SvJ background, formerly 129/SvJ. Our internal name for this line is iso129X1. C57BL/6J male mice 10–14 weeks old were mutagenized by injection with 80 mg/kg body-weight ENU (Serva) applied intraperitoneally in three weekly doses. We performed a dominant screen on F_1 animals derived from mutagenized C57BL/6J males crossed with heterozygous $Dll1^{lacZ}$ females. A minor-scale dominant screen was performed on the iso129X1 background. We injected 24–32 g heterozygous $Dll1^{lacZ}$ iso129X1 male mice intraperitoneally with four weekly doses of 50 mg/kg body weight ENU (Serva). The animals were mated to wild-type iso129X1 female mice. Breeding pairs where the first F_1

offspring came from conceptions <60 days after ENU injection were discarded to ensure that only F_1 offspring derived from ENU-exposed spermatogonal stem cell stages were analyzed for phenotyping (Soewarto *et al.* 2003). F_1 animals were genotyped and phenotyped as described below. We tested the inheritance of a phenotype by backcrossing the F_1 variants to heterozygous *Dll1*^{lacZ} mice. According to the binomial distribution (Gardner 1991), we determined with a 99.6% probability that we could isolate a mutant if we screened at least eight heterozygous *Dll1*^{lacZ} G_2 mice. Moreover, this strategy implies the isolation of phenotypes with at least 25% penetrance, which realistically enables the maintenance and further analysis of the mutant lines. Therefore, we screened at least eight heterozygous *Dll1*^{lacZ} G_2 mice and eight *Dll1*$^{+/+}$ littermates and defined a line as *Delta1* dependent if the phenotype was transmitted only to the heterozygous *Dll1*^{lacZ} mice.

Confirmed mutant lines were given names for internal use only and do not reflect official nomenclature. Mutant lines derived from our screen will be assigned with official gene symbols and names in the near future.

Genotyping: At weaning age, ~0.2-cm tail clips were gained from the animals to genotype following standardized procedures. Tail-clip samples were incubated overnight in a lysis buffer (10 mm Tris–HCl, pH 8.0, 1% (w/v) SDS, 50 mm EDTA, and 300 μg/ml proteinase K) at 57°. DNA was extracted using the Tecan Freedom Evo 150 Liquid Handling Robot and the AGOWA mag maxi DNA isolation kit. Animals were genotyped by biplex polymerase chain reaction using the primers DLL1FP 5′-CAAGGGCGTCCAGCGGTAC-3′ (which binds immediately upstream of the *Delta1* translation initiation codon) and DLL1BP 5′-CCTTGCTAGGACGCAGAGGC-3′ (which binds in exon 2), which detect a 459-bp fragment indicative of the wild-type allele, and primers Dll1FP and LacZ3BP GCACCACAGATGAAACGCCG, which detect a 222-bp fragment indicative of the mutated allele.

For linkage analysis, a genomewide mapping panel consisting of 149 single nucleotide polymorphism (SNP) markers was applied. SNPs were retrieved by using a web-based tool (ARTS; Klaften and Hrabe de Angelis 2005) and designed to be polymorphic between strains C3H/He and C3H/HeJ as a reference and BALB/cByJ and C57BL/6J for outcrossing to map the mutations generated in the Munich genomewide mutagenesis screen. Genotyping of this panel was performed using MassExtend, a matrix-assisted laser desorption/ionization time-of-flight high-throughput genotyping system supplied by Sequenom.

Dysmorphological screen: We screened the F_1 animals at weaning and again at 11 weeks of age with slight modifications of the Munich genomewide mutagenesis protocol, as described elsewhere (Fuchs *et al.* 2000). We screened for body size, body shape, right–left abnormality, skull shape, vibrissae, tooth length, tooth shape, ear size, ear form, eye size, cataracts, limbs, double digits, crippled digits, syndactylism, nails, coiled tail, general physical condition, weight, coat structure, skin color, skin structure, swellings, carcinoma/tumors, strength, gait, trembling, cramping, paralysis, seizures, respiration, general behavior, activity, aggressiveness, exploring, head tossing, head shaking, circling, landing behavior, articulation, social structure in cage, cage cleanliness, urine, and feces. The weight of the animals was determined at 6, 8, 10, and 12 weeks of age. Selected animals were analyzed with the Faxitron Specimen Radiography system model MX-20 for skeletal malformations or with the Norland-Stratec pDEXA peripheral Dual Energy X-ray between 16 and 20 weeks of age for bone density alterations.

Blood samples: We obtained blood samples (300 μl) from 12-week-old mice that had fasted overnight by puncturing the retro-orbital sinus under ether anesthesia (Hrabe de Angelis *et al.* 2000). We analyzed for clinical chemical, basic hematology, and immunological parameters in the German Mouse Clinic at the GSF Research Center (Gailus-Durner *et al.* 2005). A total of 17 clinical chemical parameters were ascertained, namely sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, creatinine, urea, cholesterol, triglycerides, aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT), alkaline phosphatase, glucose, ferritin, transferring, and lipase, as previously described (Klempt *et al.* 2006). The hematology parameters analyzed included white blood cell count, red blood cell count, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets. Additionally, the blood samples were fractionated to perform flow cytometry and ELISA analyses as described elsewhere (Kalaydjiev *et al.* 2006). The panel of immunological parameters consisted of CD4+ T cells, CD8+ T cells, γ/δ T cells, B cells (B1, B2), natural killer (NK) cells, granulocytes, monocytes, and IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, rheumatoid factor, and anti-DNA antibodies. Furthermore, all potential subpopulations that can be identified by costaining for other surface markers (IgD, B220, CD11b, MHC II, I-Ak, CD25, CD8, CD62L, CD45RA, Ly-6C, CD44) using six parameter/five color flow cytometry were analyzed.

Statistical analysis: Analysis was performed using either the software package JMP Release 5.1 (SAS Institute) or the R Version 1.12 for Mac OS X provided by the R Foundation of Statistical Computing. Outliers defined as those falling outside the 25th or 75th quantile and 1.5 times the interquantile range were discarded. The distribution of the samples was assessed using the Shapiro–Wilk normality test. If required, logarithmic transformation of the data set was performed. The variances and the means of two samples were compared using the Levene test and the two-sample *t*-test, respectively. Descriptive data are expressed as mean ± standard deviation. Variants were defined as F_1 animals showing at least one parameter differing three standard deviations from the mean template, *i.e.*, showing a *Z*-score between >3 and <−3. Every 2–3 months ~250 randomly selected F_1 samples were analyzed and a new template was generated to take into account variation due to environmental conditions already described in clinical chemical parameters (Klempt *et al.* 2006).

RESULTS

ENU mutagenesis screens for modifiers of the Delta1 phenotype: At least two breeding strategies have successfully been employed in genomewide mutagenesis programs. Mutagenized males can be crossed either to females with a different genetic background (Nolan *et al.* 2000) or to females with the same genetic background (Hrabe de Angelis *et al.* 2000). The first method has the advantage of speeding up the mapping process to identify mutations causing a variant phenotype. The second strategy makes the phenotyping more robust, as the contribution of genetic background in all progeny under study is kept constant. Therefore, we carried out both breeding strategies to screen for modifiers of the Delta1-dependent Notch signaling pathway. In the first strategy, F_1 animals were derived from mutagenized C57BL/6J males crossed to heterozygous *Dll1*^{lacZ} females in an iso129X1 background (Hrabe de Angelis *et al.* 1997), and in the second strategy, F_1 animals were

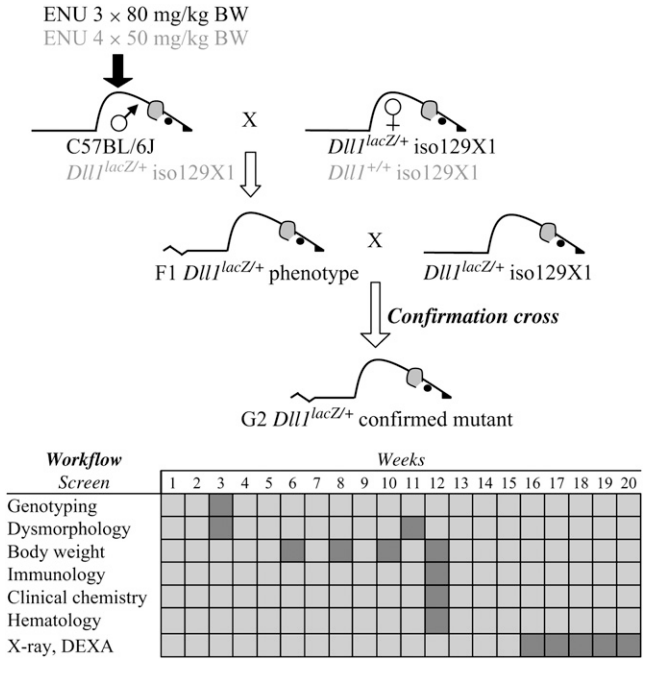

FIGURE 1.—Breeding scheme and work flow of the dominant modifier screen on Delta1. C57BL/6J or *Dll1*$^{lacZ/+}$ iso129X1 males were injected with three weekly doses of 80 mg/kg body weight or 4 weekly doses of 50 mg/kg body weight, respectively. Mutagenized C57BL/6J males were crossed to heterozygous *Dll1*$^{lacZ/+}$ iso129X1 females, and mutagenized heterozygous *Dll1*$^{lacZ/+}$ iso129X1 males were crossed to wild-type iso129X1 females. The F_1 animals underwent the phenotyping work flow depicted below; boxes with dark shading indicate the time point at which each assay was performed. Interesting heterozygous *Dll1*$^{lacZ/+}$ F_1 variants were tested for inheritance of the phenotype by backcrossing to *Dll1*$^{lacZ/+}$ iso129X1 animals. G_2 animals were then phenotyped.

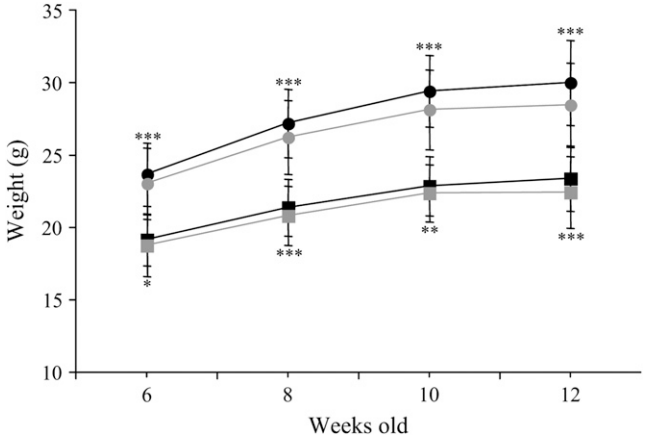

FIGURE 2.—Body-weight comparison between heterozygous *Dll1*$^{lacZ/+}$ F_1 animals and their wild-type littermates. Body weight was measured at 6, 8, 10, and 12 weeks of age. All offspring are derived from mutagenized C57BL/6J males and heterozygous *Dll1*$^{lacZ/+}$ females. Depicted are the mean ± standard deviation from wild-type F_1 males (●, $n = 288$), heterozygous *Dll1*$^{lacZ/+}$ F_1 males (■■■, $n = 324$), wild-type F_1 females (■, $n = 277$), and heterozygous *Dll1*$^{lacZ/+}$ F_1 females (●●●, $n = 299$). A two-sample *t*-test was performed comparing the weight of the animals at different ages between wild-type and heterozygous F_1 animals for male and females. *P*-values: $***P < 0.001$, $**P < 0.01$, $*P < 0.05$.

derived from mutagenized heterozygous *Dll1*^{lacZ} iso129X1 males crossed to wild-type females of the same genetic background (Figure 1). A total of 234 C57BL/6J mice were injected with 80 mg/kg ENU in three weekly doses. Each of the 92 ENU-injected males (39%) generated between 1 and 65 F_1 offspring that underwent the phenotyping work flow. Twelve ENU-treated males (5%) were excluded as the period between the last injection and the calculated time of conception was <60 days, and therefore the probability of these F_1 animals being derived from mutagenized spermatogonal stem cells was low (SOEWARTO *et al.* 2003). Moreover, 84 ENU-injected males (36%) were sterile or did not produce viable offspring. Likewise, a total of 188 heterozygous *Dll1*^{lacZ} mice were injected with 50 mg/kg ENU in four weekly doses. Sixty-three ENU males (33%) generated between 1 and 27 F_1 animals that underwent the phenotyping work flow (see Figure 1). A total of 125 ENU males were discarded because the sterility period was <60 days, they were sterile, or they did not produce any living offspring.

***Delta1* heterozygous mutant phenotypes:** To be able to identify ENU-modified phenotypes from original *Dll1*$^{lacZ/+}$ phenotypes, we characterized the phenotype of adult heterozygous *Delta1* loss-of-function mice in detail. As described previously, homozygous *Dll1*^{lacZ} mice die during embryogenesis (HRABE DE ANGELIS *et al.* 1997), and a cervical vertebrae phenotype has been described in heterozygous *Dll1*^{lacZ} animals (CORDES *et al.* 2004). We found the F_1 heterozygous *Dll1*^{lacZ} animals to be significantly lighter than their wild-type littermates (Figure 2). The data in Figure 2 are for F_1 animals, *i.e.*, the offspring of mutagenized males. Reduction of body weight in the heterozygous *Dll1*^{lacZ} animals is more pronounced in male than in female mice, but is detectable in both genders and also increases with age. These differences in body weight are also detectable in the heterozygous *Dll1*^{lacZ} iso129X1 F_1 animals when compared to their wild-type littermates (data not shown); therefore the genetic background is not important for this mutant phenotype.

At 12 weeks of age blood samples were taken from the animals by puncturing the retro-orbital sinus and several clinical chemical parameters were analyzed, as described in MATERIALS AND METHODS. Almost half of the clinical chemistry parameters were significantly altered in the F_1 heterozygous *Dll1*^{lacZ} animals compared to their wild-type littermates (Table 1). For example, serum activities of aspartate and alanine aminotransferase (AST and ALT) were increased in heterozygous *Dll1*^{lacZ} animals compared to their wild-type littermates, although the difference was significant only in males. Plasma chloride and creatinine concentrations were significantly

TABLE 1

Comparison of clinical chemical parameters between heterozygous *Dll1*^{lacZ} F_1 animals and wild-type littermates

	Male			Female		
	Wild-type F_1 ($n = 200$)	*Dll1*$^{lacZ/+}$ F_1 ($n = 187$)	*P*-value[a]	Wild-type F_1 ($n = 148$)	*Dll1*$^{lacZ/+}$ F_1 ($n = 161$)	*P*-value[a]
Sodium (mmol/liter)	152.73 ± 4.15	152.25 ± 3.52	0.23	152.44 ± 3.24	150.50 ± 4.34	<0.0001***
Potassium (mmol/liter)	4.10 ± 0.44	4.02 ± 0.53	0.11	3.95 ± 0.43	3.84 ± 0.49	0.04*
Calcium (mmol/liter)	2.09 ± 4.14	2.06 ± 0.16	0.07	2.13 ± 0.14	2.10 ± 0.17	0.048*
Cl (mmol/liter)	113.22 ± 4.29	111.43 ± 3.73	<0.0001***	113.55 ± 4.01	111.58 ± 3.39	<0.0001***
Phosphorous (mmol/liter)	1.71 ± 0.28	1.70 ± 0.31	0.76	1.72 ± 0.35	1.74 ± 0.40	0.70
Total protein (g/dl)	5.28 ± 0.41	5.23 ± 0.45	0.27	5.32 ± 0.42	5.29 ± 0.44	0.62
Creatinine (mg/dl)	0.34 ± 0.03	0.33 ± 0.03	0.04*	0.35 ± 0.03	0.34 ± 0.03	0.04*
Urea (mg/dl)	51.28 ± 8.49	47.40 ± 8.48	<0.0001***	45.30 ± 7.51	45.13 ± 8.61	0.86
Cholesterol (mg/dl)	125.01 ± 16.38	123.20 ± 16.16	0.28	102.66 ± 16.67	104.56 ± 15.94	0.31
Triglyceride (mg/dl)	113.40 ± 3.41	107.13 ± 36.55	0.08	90.20 ± 23.95	95.52 ± 28.80	0.08
ALT (unit/liter)	12.76 ± 3.67	13.85 ± 4.28	0.01*	12.44 ± 2.80	13.03 ± 3.88	0.14
AST (unit/liter)	21.90 ± 4.83	24.06 ± 6.97	0.0006***	24.06 ± 4.80	24.38 ± 5.31	0.58
Alkaline phosphatase (unit/liter)	68.26 ± 15.36	67.79 ± 13.61	0.75	108.40 ± 21.41	101.51 ± 24.85	0.009**
Glucose (mg/dl)	152.33 ± 45.62	136.61 ± 55.02	0.002**	151.95 ± 47.97	142.42 ± 47.67	0.08
Ferritin (ng/dl)	30.95 ± 10.38	27.56 ± 10.21	0.002**	28.38 ± 9.26	28.43 ± 8.91	0.97
Transferrin (mg/dl)	149.97 ± 7.32	148.86 ± 6.44	0.13	151.51 ± 6.52	149.86 ± 6.76	0.035*
Lipase (unit/liter)	48.02 ± 9.88	47.32 ± 14.51	0.60	53.14 ± 10.84	56.13 ± 12.41	0.03*

All F_1 offspring were derived from mutagenized C57BL/6J and heterozygous *Dll1*^{lacZ} females.
[a] *P*-value from two-sample *t*-test: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

decreased in both male and female heterozygous *Dll1*^{lacZ} animals. In the iso129X1 background, 65% of the examined parameters were significantly altered and most of them showed the tendency to be decreased except for ALT and AST (Table 2). All these differences were of course taken into account when screening for ENU-induced phenotypic variants in the dominant modifier screens. The genetic background of a mouse strain has

TABLE 2

Comparison of clinical chemical parameters between male F_1 animals depending on their genetic background

	Dll1$^{lacZ/+}$ F_1			Wild-type F_1		
	SV7[a] ($n = 344$)	129X1_DL1[b] ($n = 256$)	*P*-value[c]	SV7[a] ($n = 340$)	129X1_DL1[b] ($n = 143$)	*P*-value[c]
Sodium (mmol/liter)	150.13 ± 4.81	151.66 ± 4.66	0.0002***	150.29 ± 5.46	153.14 ± 4.05	<0.0001***
Potassium (mmol/liter)	3.93 ± 0.45	4.37 ± 0.51	<0.0001***	4.01 ± 0.44	4.39 ± 0.56	<0.0001***
Calcium (mmol/liter)	2.03 ± 0.14	2.07 ± 0.15	0.004**	2.05 ± 0.14	2.12 ± 0.13	0.0483*
Cl (mmol/liter)	110.00 ± 4.06	111.37 ± 3.80	<0.0001***	111.32 ± 4.70	112.72 ± 3.63	<0.0001***
Phosphorous (mmol/liter)	1.70 ± 0.31	1.76 ± 0.29	0.003**	1.68 ± 0.30	1.66 ± 0.34	<0.0001***
Total protein (g/dl)	5.25 ± 0.43	5.17 ± 0.39	0.028*	5.27 ± 0.42	5.26 ± 0.41	0.78
Creatinine (mg/dl)	0.32 ± 0.04	0.31 ± 0.03	0.32	0.32 ± 0.04	0.32 ± 0.02	0.40
Urea (mg/dl)	46.37 ± 7.70	42.93 ± 9.96	<0.0001***	49.63 ± 8.94	47.95 ± 11.04	0.12
Cholesterol (mg/dl)	117.51 ± 15.70	135.79 ± 25.56	<0.0001***	119.72 ± 16.82	140.60 ± 26.95	<0.0001***
Triglyceride (mg/dl)	108.72 ± 37.80	110.75 ± 42.72	0.56	119.12 ± 37.02	136.14 ± 44.92	0.0001***
ALT (unit/liter)	12.51 ± 4.01	11.17 ± 3.33	<0.0001***	11.26 ± 2.89	10.78 ± 3.03	0.11
AST (unit/liter)	23.69 ± 6.73	23.59 ± 5.07	0.84	21.44 ± 4.70	23.42 ± 5.03	<0.0001***
Alkaline phosphatase (unit/liter)	66.25 ± 12.36	68.47 ± 15.01	0.06	67.03 ± 12.85	73.10 ± 18.68	0.0005***
Glucose (mg/dl)	124.44 ± 51.85	106.36 ± 35.62	<0.0001***	139.41 ± 46.36	118.43 ± 41.61	<0.0001***
Ferritin (ng/dl)	31.43 ± 10.04	36.48 ± 13.32	<0.0001***	31.87 ± 8.64	37.26 ± 16.81	0.0004***
Transferrin (mg/dl)	150.36 ± 10.36	146.62 ± 9.03	<0.0001***	152.21 ± 10.50	148.86 ± 10.57	0.002**
Lipase (unit/liter)	44.53 ± 12.38	43.99 ± 10.21	0.58	46.10 ± 9.60	43.54 ± 11.84	0.03*

[a] F_1 animals were derived from mutagenized C57BL/6J and heterozygous *Dll1*^{lacZ}-iso129X1 females.
[b] F_1 animals were derived from mutagenized heterozygous *Dll1*^{lacZ}-iso129X1 males and iso129X1 females.
[c] *P*-value from two-sample *t*-test: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

TABLE 3

F_1 animals screened in the two Delta1 dominant modifier genetic screens

	Breeding pair		
	C57BL/6J[a]/*Dll1*^{lacZ} iso129X1	*Dll1*^{lacZ} iso129X1[a]/iso129X1	Total
Total F_1 produced	1673	609	2282
	Dysmorphological screen		
F_1 *Dll1*$^{lacZ/+}$	708	303	1011
F_1 wild type	934	272	1206
CC[b] set up	26	4	30
Mutant lines total	4	0	4
Mutant lines *Delta1* dependent	0	0	0
	Immunological screen		
F_1 *Dll1*$^{lacZ/+}$	676	284	960
F_1 wild type	0	0	0
CC[b] set up	11	15	26
Mutant lines total	7	4	11
Mutant lines *Delta1* dependent	1	1	2
	Clinical chemical screen		
F_1 *Dll1*$^{lacZ/+}$	672	247	919
F_1 wild type	630	134	764
CC[b] set up	60	10	70
Mutant lines total	18	2	20
Mutant lines *Delta1* dependent	5	0	5

[a] Male injected with ENU.
[b] CC, confirmation cross.

strong influence on clinical parameters (see Mouse Phenome Database at the Jackson Laboratory: http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home). As seen in Table 2, >75% of the clinical chemical parameters that were identified as components of the *Dll1*$^{lacZ/+}$ phenotype were significantly different compared to the F_1 animals derived from mutagenized C57BL/6J or heterozygous *Dll1*^{lacZ} iso129X1 males. Therefore, when phenotyping for variants, different reference values were used, depending on the appropiate genetic background. In male animals, only creatinine and AST were influenced by the genotype, but not by the genetic background investigated.

iso129X1 genetic background: The dysmorphology protocol was designed to screen C3HeB/FeJ animals in the Munich ENU genomewide mutagenesis screen (Fuchs *et al.* 2000; Hrabe de Angelis *et al.* 2000). We had to modify this protocol as described in materials and methods, as some of the parameters, such as the Preyer reflex and coat color, were variable among animals of the iso129X1 background. To evaluate the isogenicity of the background, we used the SNP panel developed at our institute to map mutant alleles from the genomewide Munich mutagenesis screen, as described in materials and methods. For this purpose, 15 wild-type and 18 heterozygous *Dll1*^{lacZ} mice from three different generations of our iso129X1 breeding colony were genotyped. Of the 137 SNP assays analyzed, 30% showed heterozygosity (data not shown). This is probably one of the main reasons for the variable phenotypes found by analyzing some parameters. The polymorphic genotype is most probably due to the backcrossing strategy of 129 substrains, which have a high degree of genetic variation (Simpson *et al.* 1997; Threadgill *et al.* 1997). Due to this fact, the nomenclature of the 129 substrains has been revised recently (Festing *et al.* 1999).

Dysmorphological screen: We have screened >2200 F_1 animals in the search for modifiers of the *Dll1*$^{lacZ/+}$ phenotype (Table 3). Due to the role of the Delta/Notch pathway in the development of the mesoderm, we screened for morphological variants (Fuchs *et al.* 2000). Depending on the breeding strategy of the modifier genetic screen, 3.6% (26 animals derived from C57BL/6J mutagenized males) or 1.3% (four animals derived from iso129X1 mutagenized males) of the heterozygous *Dll1*^{lacZ} F_1 animals tested (see Table 3) showed variant phenotypes absent in their wild-type littermates and the *Dll1*$^{lacZ/+}$ reference population.

The F_1 variants were backcrossed for testing the coinheritance of the phenotype with heterozygous *Dll1*^{lacZ} mice as described in Figure 1. At present, seven of these confirmation crosses are still running, another seven have failed because the F_1 animals were sterile or died before the screen was finished, 12 variant phenotypes were not transmitted, and four were confirmed but were shown to be independent of the disruption of the *Delta1* allele. All the mutant lines generated to this point are

TABLE 4

Mutant lines generated in Delta1 modifier dominant screen

Line name	Variant phenotype	Penetrance[a] (%)	Background	β[b] (%)
	Delta1-dependent mutant lines			
DCO001	High cholesterol	44	SV7	0.8
DFE001	Altered iron metabolism parameters	58	SV7	11
DFE002	Altered iron metabolism parameters	42	SV7	12
DLI001	High AST	27	SV7	3
DTP001	Altered kidney metabolism parameters	40	SV7	20
TUB047	DX5 high	50	129X1_DL1	12
TUB060	CD8 high	50	SV7	7
	Delta1-independent mutant lines			
BCC007	Different hair lengths	50	SV7	
D1379	Altered basic hematology parameters	24	SV7	
D1380	Altered bone parameters	20	SV7	
D1534	Altered kidney metabolism parameters	40	SV7	
D1679	Curly coat	53	SV7	
DHT001	High urea	22	129X1_DL1	
LIV002	High AST, ALT	100	SV7	
LIV003	High AST, ALT	22	SV7	
SCK001	High creatine kinase	100	SV7	
SCO001	High cholesterol	100	SV7	
SCO002	High glucose and cholesterol	23	SV7	
SCO003	High cholesterol	20	SV7	
SFE002	High transferrin	10	SV7	
SFR002	High transferrin and iron	93	SV7	
SPA002	High lipase	40	SV7	
SPA003	High lipase and amylase	50	129X1_DL1	
SPO001	High alkaline phosphatase	12	SV7	
SWE001	Lighter	32	SV7	
SXR001	One rib pair missing	100	SV7	
TUB014	Low B cells	32	SV7	
TUB022	CD3 high, CD19 low	23	SV7	
TUB023	CD8 high	70	SV7	
TUB040	CD3 low	25	SV7	
TUB046	CD19 low	43	129X1_DL1	
TUB056	CD8 low	71	129X1_DL1	
TUB059	DX5, CD8 high	58	SV7	
TUB062	CD19 low	11	SV7	
TUB063	CD19 low	50	129X1_DL1	

[a] Only heterozygous animals were considered if transmitted only to *Dll1*^{lacZ} animals.
[b] Remaining probability not to be a *Dll1*-dependent line is given by the binomial distribution.

listed in Table 4.The four *Delta1*-independent mutant lines generated in the dysmorphology screen display distinct phenotypes. A mutant line, SXR001, has been detected by performing X-ray analysis. In these animals one thoracic rib pair is missing. The other mutant phenotypes include alterations of coat shape such as curly coat (D1679) or coat length (BCC007) or reduction of the body weight (SWE001).

Immunological screen: The Notch signaling pathway plays a crucial role in the differentiation of lymphoid to B or T cells and within the αβT cell lineage in differentiating to either helper or cytotoxic T cells (Allman *et al.* 2002; Radtke *et al.* 2004, 2005). For this reason, we analyzed B- and T-cell markers in peripheral blood. A total of 960 heterozygous *Dll1*^{lacZ} F_1 animals have been screened at present (Table 3). In case of a variation in a parameter, the heterozygous *Dll1*^{lacZ} F_1 animal and its wild-type littermate were sampled a second time to confirm that presence of the variant phenotype was restricted to the heterozygous *Dll1*^{lacZ} mouse. Of the heterozygous *Dll1*^{lacZ} F_1 animals deriving from ENU-mutagenized C57BL/6J males, 1.6% (11 animals) showed variant phenotypes absent in their wild-type littermates and the *Dll1*$^{lacZ/+}$ mice reference population. These included lower CD19-positive B-cell values and higher CD8-positive T-cell values. In addition, when analyzing F_1 animals derived from mutagenized *Dll1*^{lacZ} iso129X1, 5.3% (15 animals) showed an alteration of immunological parameters. Four confirmation crosses are still running, nine failed because the F_1 animal died

before the screen was finished, one alteration was not transmitted, and one was transmitted, but its dependency on the disruption of the *Delta1* locus still has to be determined. Nine were confirmed but were found not to be *Delta1* dependent. However, two mutant lines showed a *Delta1*-dependent transmission of the phenotype (Table 3). The *Delta1*-independent mutant lines display a variety of phenotypes affecting immunological parameters. These include altered T-cell markers, such as in the mutant line TUB059; altered B-cell markers, such as in the mutant line TUB063; or alterations of both markers, such as in the mutant line TUB022 (Table 4).

Of highest interest for our goal of identifying modifiers of the Notch signaling pathway are those lines that show co-inheritance of a variant phenotype only in the heterozygous $Dll1^{lacZ}$ G_2 animals but not in their wild-type littermates. There is always a remaining probability for finding wild-type animals showing the observed phenotype. This probability (β) is dependent upon the penetrance of the phenotype and the number of wild-type animals screened and is given by the binomial distribution (see Table 4). As already mentioned, two immunological mutant lines that interact with the heterozygous *Delta1* knockout allele have been established. $TUB047^{+/-}/Dll1^{lacZ/+}$ animals showed alterations in the differentiation of lymphoid and show higher levels of DX5-positive cells in blood, a marker for natural killer cells. Of the 13 G2 $TUB047/Dll1^{lacZ/+}$ animals screened, 3 showed the variant phenotype, whereas none of the eight $TUB047/Dll1^{+/+}$ showed alteration of DX5-positive cells levels in blood. In the other immunological mutant line established, the $TUB060^{+/-}/Dll1^{lacZ/+}$ animals presented higher levels of CD8-positive T cells in blood. In this case, 3 of the 12 G_2 $TUB060/Dll1^{lacZ/+}$ animals screened showed the variant phenotype, whereas none of the 10 $TUB60/Dll1^{+/+}$ showed alteration of CD8-positive cell levels in blood. Both mouse lines show a penetrance of 50%; *i.e.*, 25% of the heterozygous $Dll1^{lacZ}$ G_2 animals show the phenotype. These lines are being further phenotypically characterized and backcrossed to the iso129X1 background. Intercrosses will further confirm the dependency of the phenotype on the disruption of the *Delta1* locus.

Clinical chemical screen: Because the Notch signaling pathway is involved in numerous biological processes, we also screened for clinical chemical and hematological parameters, which provide a powerful diagnostic profile to detect defects in various organ systems and changes in numerous metabolic pathways. A total of 919 heterozygous $Dll1^{lacZ}$ F_1 animals and 764 wild-type littermates have been screened so far (Table 3). As mentioned in MATERIALS AND METHODS, some parameters differ, depending on the genetic background or disruption of the *Delta1* locus. These differences were taken into account when searching for variants. In case of a variation in a parameter, a second blood sample was taken 2 weeks later to confirm the phenotypic variation. For testing the inheritance of the phenotype with heterozygous $Dll1^{lacZ}$ mice, 8.9% (60 of 672 animals) and 4% (10 of 247 animals) of the heterozygous $Dll1^{lacZ}$ F_1 were backcrossed (Table 3). Seventeen confirmation crosses are still running, 14 failed because the F_1 animals were sterile or died before the screen was finished, and 17 crosses showed no inheritance of the phenotype. Two variant phenotypes were inherited but the dependency on the disruption of the *Delta1* locus still has to be determined. Fifteen variant phenotypes were confirmed but were *Delta1* independent and five mutant lines showed a *Delta1*-dependent transmission of the phenotype. The *Delta1*-independent mutant lines established by investigating clinical chemical parameters cover distinct areas of metabolism and organ function (Table 4), affecting pancreatic metabolism (SPA003 mutant line), cholesterol levels (SCO003 mutant line), liver metabolism (LIV002 mutant line), iron metabolism (SFR002 mutant line), and kidney function (D1534 mutant line). One line (D1379) has been established with altered basic hematological parameters. Most of the founders and mutant G_2 animals of all the established lines have been cryopreserved as described before (MARSCHALL and HRABE DE ANGELIS 1999) for further analysis by the scientific community.

Of major interest are the five mutant lines where the clinical chemical parameters are altered only in conjunction with inheritance of one disrupted allele of the *Delta1* locus. In the DCO001 mutant line, affected animals show higher plasma cholesterol levels. Of the 19 G_2 $DCO001/Dll1^{lacZ/+}$ animals screened, 4 showed the variant phenotype, whereas none of the 23 $DCO001/Dll1^{+/+}$ showed alteration in the cholesterol levels. In the DFE001 and DFE002 mutant lines, the animals show alterations in plasma transferrin, ferritin, and/or iron levels. Of 19 G_2 $DFE002/Dll1^{lacZ/+}$ animals, 4 showed altered levels of transferrin in blood, whereas none of the 10 $DFE002/Dll1^{+/+}$ showed the phenotype. In the DLI001 mutant line, the animals show high aspartate aminotransferase activities. Of 45 G_2 $DLI001/Dll1^{lacZ/+}$ animals, 6 showed the pehnotype, whereas none of the 26 $DLI001/Dll1^{+/+}$ showed high aspartate aminotransferase activities. $DLI001^{+/-}$ G_2 mutant animals were backcrossed to C57BL/6J, and the phenotype was recovered exclusively in heterozygous *Delta1* mutant animals. Animals of the DTP001 mutant line show alterations in plasma creatinine, total protein, and electrolyte concentrations. In $DTP001^{+/-}$ animals, the phenotype expressivity varies, showing high levels of creatinine, total protein, and/or electrolytes in blood. Of 15 G_2 $DTP001/Dll1^{lacZ/+}$ animals, 3 showed the phenotype, whereas none of the 8 $DTP001/Dll^{+/+}$ animals screened showed it. We backcrossed mutant G_2 animals to iso129X1 animals and only heterozygous $Dll1^{lacZ}$ mutant animals showed either high total protein or high creatinine levels.

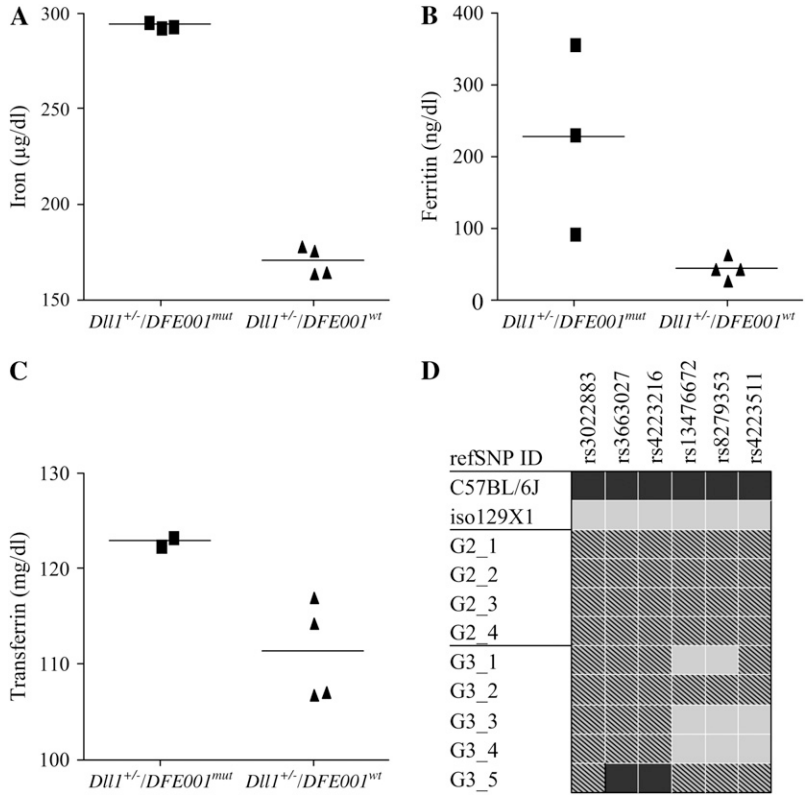

FIGURE 3.—Characterization of the Delta1-dependent mutant line DFE001. (A) Iron levels in blood from heterozygous *Dll1*^{lacZ} DFE001 mutant and wild-type G_2 females. (B) Ferritin in blood from heterozygous *Dll1*^{lacZ} DFE001 mutant and wild-type G_3 males, derived from an intercross with two G_2 DFE001 mutant animals. (C) Transferrin in blood from heterozygous *Dll1*^{lacZ} DFE001 mutant and wild-type G_3 males. (D) Rough mapping of the DFE001 mouse line. SNP markers for chromosome 2 are shown. Four G_2 and five G_3 DFE001 mutant animals were genotyped. Solid boxes, the C57BL/6J allele; shaded boxes, the iso129X1 allele; hatched boxes, heterozygosity.

The DFE001 mutant line displays a complex phenotype, with animals showing altered values of transferrin, ferritin, and/or iron. Of the 24 G_2 *DFE001/Dll1*$^{lacZ/+}$ animals screened, 2 showed increased transferrin levels in blood and 5 showed increased iron levels, whereas none of the 7 G_2 *DFE001/Dll1*$^{+/+}$ animals screened showed alterations in any of these parameters. In Figure 3A the female *Dll1*$^{lacZ/+}$ mutant animals showing higher levels of iron in blood are depicted. These animals showed a *Z*-score of 3–4.9 of the iron levels. A similar effect was observed in male *Dll1*$^{lacZ/+}$ mutants; one of the compound mutants showed higher iron levels (*Z*-score = 3.6) and the other higher transferrin levels in blood (*Z*-score = 3.2). Moreover, only heterozygous *Dll1*^{lacZ} G_3 mice born from an intercross showed the mutant phenotype. As shown in Figure 3B, the ferritin levels were increased in three *Dll1*$^{lacZ/+}$ mutants showing a *Z*-score of 4–26 from the mean of the heterozygous *Dll1*^{lacZ} G_3 wild-type littermates. The other two *Dll1*$^{lacZ/+}$ mutant animals showed higher transferrin levels than their heterozygous *Dll1*^{lacZ} G_3 wild-type littermates, with a Z-score of 3.1 and 3.3 (Figure 3C). The later compound G_3 mutant also showed higher iron levels in blood (*Z*-score = 3). We carried out a preliminary rough mapping of the mutation of DFE001, using our SNP platform mentioned above. Four G_2 *Dll1*$^{lacZ/+}$ mutant animals and five G_3 *Dll1*$^{lacZ/+}$ mutant animals were genotyped. The DFE001 mutation seems to show a linkage to the proximal region of the marker rs3663027 (57.5 Mb, *Mus musculus* genome build 36.1) on chromosome 2, with marker rs3022883 (37.5 Mb, *M. musculus* genome build 36.1) having only parental alleles, as shown in Figure 3D. *Notch1* is located 11 Mb proximal to rs3022883. Therefore, this *Notch* receptor gene was the obvious candidate to sequence. We sequenced the coding region and exon/intron boundaries of *Notch1* and no change compared to the parental sequence could be detected. Therefore, the DFE001 mutant line may reveal a novel modifier of Delta1 or the mutation could occur in a *cis*-regulatory element of *Notch1*.

DISCUSSION

Role of genetic background in the *Delta1* modifier screen: Modifiers of the Notch signaling pathway still need to be elucidated to explain all the biological functions and malfunctions in which this pathway is involved. Delta1 is one of the recognized ligands of Notch receptors. Thus we are carrying out a dominant modifier screen combining forward and reverse genetics to elucidate novel modifiers of Delta1 by mating ENU-mutagenized mice and heterozygous *Dll1*^{lacZ} mice. In our screen, both C57BL/6J and heterozygous *Dll1*^{lacZ} males were mutagenized with ENU and mated with females to generate F_1 animals. Mutagenesis of heterozygous *Dll1*^{lacZ} mice for the production of F_1 animals was less efficient than mutagenesis of C57BL/6J mice, as already described (WEBER *et al.* 2000). The reduced breeding performance of the iso129X1 background could be due to greater susceptibility to ENU if one

allele of the *Delta1* locus is missing. The reduced body weight may also contribute to the reduced fertility.

The genetic variation due to contamination of the 129X1/SvJ substrain, formerly named 129/SvJ, has recently been described in detail (Simpson *et al.* 1997; Threadgill *et al.* 1997). Therefore, a genetic monitoring of the background was required and we determined genetic heterozygosity using a SNP panel.

Novel phenotypes in heterozygous *Dll1^{lacZ}* animals: Homozygous *Dll1^{lacZ}* animals die around embryonic day 12.5 (Hrabe de Angelis *et al.* 1997), and a homeotic transformation in the cervical region has been described in heterozygous *Dll1^{lacZ}* animals (Cordes *et al.* 2004). Here we showed that disruption of the *Delta1* locus in one allele also leads to reduced body weight in the mutagenized offspring, regardless of age, gender, or background. Moreover, several clinical chemical parameters are also altered in mutagenized *Dll1$^{lacZ/+}$* offspring. Chloride is significantly decreased in heterozygous *Dll1^{lacZ}* animals compared to their wild-type littermates. In addition, creatinine was decreased in blood in heterozygous animals regardless of gender. Both findings indicate that kidney metabolism is altered in these mice when compared to their littermates. Further analyses are required to understand the underlying mechanisms of these differences between heterozygous and wild-type mutagenized offspring.

Morphology and the Notch signaling pathway: Because the Notch signaling pathway plays a crucial role in morphogenesis, we screened for dysmorphological variations following a protocol that has been shown to be successful in our laboratory for identifying a broad range of morphological variations (Vreugde *et al.* 2002; Fitch *et al.* 2003; Hafezparast *et al.* 2003). Although four mutant lines have been established in our screen, we have not yet found any dysmorphological variant whose phenotype was dependent on the disruption of the *Delta1* locus. A possible explanation is the background used, 129X1/SvJ, which shows high variability in morphology; therefore we had to adapt our protocol. Other probable explanations are that the majority of mutants could be lethal during embryonic development due to the essential role of the Notch signaling pathway in the first days of development or that recessive modifiers are required to detect variations in the phenotype. Nevertheless, at least one exception has already been described. Heterozygous *Dll1^{lacZ}* mice have been shown to be an enhancer of the rib–vertebrae phenotype (Beckers *et al.* 2000). Further studies revealed *Tbx6* to be the gene mutated in this animal model (Watabe-Rudolph *et al.* 2002) and to be an interactor of the Notch signaling pathway, playing a major role in somitogenesis (White *et al.* 2003; Yasuhiko *et al.* 2006). Certainly more modifiers of the Notch signaling pathway could affect morphology. We will therefore carry on our screen focusing on bone alterations. Cell culture studies indicate a role of the Notch signaling pathway in bone morphogenesis, but with contradictory proposed functions. Some postulate inhibition of osteoblastogenesis (Deregowski *et al.* 2006) and others induction of osteoblast differentiation (Nobta *et al.* 2005).

Immunological phenotypes of the Notch signaling pathway: Notch signaling plays a major role in cell-fate decision in the immune system, mainly in T-cell differentiation and activation (Allman *et al.* 2002; Radtke *et al.* 2004, 2005; Tsukumo and Yasutomo 2004). Conditional ablation of floxed Notch1 alleles, induced by interferon-regulated creatinine–recombinase expression, produced an increase of B cells and a decrease of T cells in the thymus (Radtke *et al.* 1999; Wilson *et al.* 2001). In contrast, conditional disruption of the *Delta1* locus in lymphoid tissue revealed an unexpected phenotype, having no effect on T cells but an effect on the generation of marginal B cells (Hozumi *et al.* 2004). A more recent study has shed further light on the effect of Notch signaling, showing that it can influence the differentiation of immature thymocytes into the NK cell and γδT-cell lineages during a brief window of development between the DN1 and DN3 stages (Lehar *et al.* 2005). Therefore, different questions still have to be resolved to explain the mechanism of cell fate in the immune system. In our screen we successfully identified two compound mutant lines in which the immunological phenotype was dependent on the disruption of the *Delta1* locus and the presence of an ENU mutation. Interestingly, both lines show alterations in lymphocyte development in blood or in T-cell maturation. Both derived from independent ENU-injected males, and therefore most probably harbor different mutations. These lines will provide a powerful *in vivo* tool to investigate cell-fate decision in the immune system.

Novel metabolic phenotypes of the Notch signaling pathway: Studies of *Delta1* expression in adulthood are scarce. In mice, *Delta1* expression has been detected by Northern blot in heart and lung (Bettenhausen *et al.* 1995) and in humans faintly in several tissues such as heart, lung, kidney, and muscle (Gray *et al.* 1999). Moreover, information about the role of Delta1 in adulthood is scarce, and no disease has yet been linked to this ligand (Harper *et al.* 2003). We searched for modifiers of Delta1 by screening for clinical chemical parameters, which provide a powerful diagnostic tool to detect defects resulting in alterations of various organ systems and changes in numerous metabolic pathways. As mentioned before, 21 mutant lines that demonstrate again the effectiveness of the screen have been established. More interesting for our purpose are the 5 mutant lines established that show phenotypes that are dependent on the presence of a disrupted copy of the *Delta1* gene. The phenotypes cover different aspects of metabolism. A link between cholesterol and Delta1 may be explained by the α-secretase ADAM10. ADAM10-deficient mice die at embryonic day 9.5, and these mice

showed a higher expression of *Delta1* (Hartmann *et al.* 2002). On the other hand, cell culture studies have demonstrated that low cholesterol levels promote the nonamyloidogenic pathway by increasing ADAM10 expression (Kojro *et al.* 2001). Further analysis is required to investigate why cholesterol levels are higher in the DCO001 mutant animals and which kind of interaction exists with Delta1. The DLI001 mutant animals show higher levels of AST in blood. AST is usually elevated in blood in response to liver tissue damage. This effect is most probably secondary to another dysfunction and therefore more animals will be required for further analyses. Interesting are alterations in kidney metabolism; as already mentioned, only one electrolyte and creatinine levels seem to be altered in heterozygous *Dll1*^{lacZ} mice regardless of background or gender. Notch signaling plays a key role in kidney development and repair of tissue damage, but genes other than Delta1 seem to be involved in this process, too, such as Notch2 and Jagged1 (McCright 2003). Nevertheless, *Delta1* is expressed in kidney and our screen has provided a mutant line, DTP001, where a potential modifier of Delta1 induces an increase of creatinine and other kidney metabolites in blood. Clarifying which ENU-mutated gene is responsible for this phenotype will help to elucidate the role of Delta1 in the kidney in adulthood. The role of Notch signaling in iron metabolism is not known. We have established two compound mutant lines with alterations in iron metabolites. DFE001 animals have a mutation that leads to modification of the heterozygous phenotype increasing ferritin levels in blood. We preliminary mapped the mutation to chromosome 2, but *Notch1* does not seem to be the mutated gene. Therefore, DFE001 may reveal a novel modifier of the Notch signaling pathway or the mutation could occur in a *cis*-regulatory element of *Notch1*. Moreover, 31 confirmation crosses that will deliver more interesting mutant lines are still running.

Conclusion: Here we present the mutagenesis screens running at our laboratory using *Delta1* heterozygous animals with the main focus on searching for modifiers of the Notch signaling pathway. We have shown that heterozygous *Dll1*^{lacZ}-mutagenized offspring show a reduction of body weight and alterations in clinical chemical parameters compared to their wild-type littermates. We have established 35 mutant lines that consolidate the genomewide mutagenesis project that has been run at our laboratory for several years. Of major interest are the 7 new mutant lines that might reveal new modifiers of the Notch signaling pathway. Therefore, new mouse models have been established to understand the role of Notch signaling in liver, kidney, iron, and cholesterol metabolism and in cell-fate decision in adulthood in the immune system.

We kindly thank Mike Atkinson for editing, Veronique Blanquet and Jordi Guimera for scientific discussion, and Susan Marschall, Auke Boersman, Monika Beschorner, and Stefanie Dunst for the cryopreservation of the lines. We kindly thank Gerlinde Bergter, Silvia Crowley, Christine Fürmann, Sandra Hoffmann, Elfi Holupirek, Kerstin Kutzner, Nadine Lindemann, Andreas Mayer, Marcel Schieven, Michael Schulz, and Magdalena Trochimiuk for technical assistance. This work was supported by the Nationale Genomforschungsnetz (grant nos. 01GR0430 and 01GR0434).

LITERATURE CITED

Allman, D., J. C. Aster and W. S. Pear, 2002 Notch signaling in hematopoiesis and early lymphocyte development. Immunol. Rev. **187:** 75–86.

Apelqvist, A., H. Li, L. Sommer, P. Beatus, D. J. Anderson *et al.*, 1999 Notch signalling controls pancreatic cell differentiation. Nature **400:** 877–881.

Artavanis-Tsakonas, S., M. D. Rand and R. J. Lake, 1999 Notch signaling: cell fate control and signal integration in development. Science **284:** 770–776.

Balling, R., 2001 ENU mutagenesis: analyzing gene function in mice. Annu. Rev. Genomics Hum. Genet. **2:** 463–492.

Beckers, J., N. Schlautmann and A. Gossler, 2000 The mouse ribvertebrae mutation disrupts anterior-posterior somite patterning and genetically interacts with a Delta1 null allele. Mech. Dev. **95:** 35–46.

Bettenhausen, B., M. Hrabe de Angelis, D. Simon, J. L. Guenet and A. Gossler, 1995 Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. Development **121:** 2407–2418.

Bianchi, S., M. T. Dotti and A. Federico, 2006 Physiology and pathology of notch signalling system. J. Cell. Physiol. **207:** 300–308.

Brooker, R., K. Hozumi and J. Lewis, 2006 Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. Development **133:** 1277–1286.

Bulman, M. P., K. Kusumi, T. M. Frayling, C. McKeown, C. Garrett *et al.*, 2000 Mutations in the human delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. Nat. Genet. **24:** 438–441.

Carpinelli, M. R., D. J. Hilton, D. Metcalf, J. L. Antonchuk, C. D. Hyland *et al.*, 2004 Suppressor screen in Mpl−/− mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. Proc. Natl. Acad. Sci. USA **101:** 6553–6558.

Cordes, R., K. Schuster-Gossler, K. Serth and A. Gossler, 2004 Specification of vertebral identity is coupled to Notch signalling and the segmentation clock. Development **131:** 1221–1233.

Cormier, R. T., A. Bilger, A. J. Lillich, R. B. Halberg, K. H. Hong *et al.*, 2000 The Mom1AKR intestinal tumor resistance region consists of Pla2g2a and a locus distal to D4Mit64. Oncogene **19:** 3182–3192.

Curtis, D. J., 2004 Modifier screens in the mouse: time to move forward with reverse genetics. Proc. Natl. Acad. Sci. USA **101:** 7209–7210.

Deregowski, V., E. Gazzerro, L. Priest, S. Rydziel and E. Canalis, 2006 Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenetic protein signaling. J. Biol. Chem. **281:** 6203–6210.

Dunwoodie, S. L., D. Henrique, S. M. Harrison and R. S. Beddington, 1997 Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. Development **124:** 3065–3076.

Ellisen, L. W., J. Bird, D. C. West, A. L. Soreng, T. C. Reynolds *et al.*, 1991 TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell **66:** 649–661.

Festing, M. F., E. M. Simpson, M. T. Davisson and L. E. Mobraaten, 1999 Revised nomenclature for strain 129 mice. Mamm. Genome **10:** 836.

Fitch, K. R., K. A. McGowan, C. D. van Raamsdonk, H. Fuchs, D. Lee *et al.*, 2003 Genetics of dark skin in mice. Genes Dev. **17:** 214–228.

Fuchs, H., K. Schughart, E. Wolf, R. Balling and M. Hrabe de Angelis, 2000 Screening for dysmorphological abnormalities: a powerful tool to isolate new mouse mutants. Mamm. Genome **11:** 528–530.

Gailus-Durner, V., H. Fuchs, L. Becker, I. Bolle, M. Brielmeier *et al.*, 2005 Introducing the German Mouse Clinic: open access platform for standardized phenotyping. Nat. Methods **2:** 403–404.

Gardner, E. J., M. J. Simmons and D. P. Snustad, 1991 *Principles of Genetics*. John Wiley & Sons, New York.

Gray, G. E., R. S. Mann, E. Mitsiadis, D. Henrique, M. L. Carcangiu *et al.*, 1999 Human ligands of the Notch receptor. Am. J. Pathol. **154:** 785–794.

Hafezparast, M., R. Klocke, C. Ruhrberg, A. Marquardt, A. Ahmad-Annuar *et al.*, 2003 Mutations in dynein link motor neuron degeneration to defects in retrograde transport. Science **300:** 808–812.

Harper, J. A., J. S. Yuan, J. B. Tan, I. Visan and C. J. Guidos, 2003 Notch signaling in development and disease. Clin. Genet. **64:** 461–472.

Hartmann, D., B. de Strooper, L. Serneels, K. Craessaerts, A. Herreman *et al.*, 2002 The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum. Mol. Genet. **11:** 2615–2624.

Hozumi, K., N. Negishi, D. Suzuki, N. Abe, Y. Sotomaru *et al.*, 2004 Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat. Immunol. **5:** 638–644.

Hrabe de Angelis, M., J. McIntyre, II and A. Gossler, 1997 Maintenance of somite borders in mice requires the Delta homologue DII1. Nature **386:** 717–721.

Hrabe de Angelis, M., H. Flaswinkel, H. Fuchs, B. Rathkolb, D. Soewarto *et al.*, 2000 Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat. Genet. **25:** 444–447.

Jarriault, S., C. Brou, F. Logeat, E. H. Schroeter, R. Kopan *et al.*, 1995 Signalling downstream of activated mammalian Notch. Nature **377:** 355–358.

Joutel, A., C. Corpechot, A. Ducros, K. Vahedi, H. Chabriat *et al.*, 1996 Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature **383:** 707–710.

Kadesch, T., 2004 Notch signaling: the demise of elegant simplicity. Curr. Opin. Genet. Dev. **14:** 506–512.

Kalaydjiev, S., T. Franz and D. Busch, 2006 Mouse phenotyping: immunology, pp. 237–252 in *Standards of Mouse Model Phenotyping*, edited by M. Hrabé de Angelis, P. Chambon and S. Brown. Wiley-VCH Verlag, Germany.

Klaften, M., and M. Hrabe de Angelis, 2005 ARTS: a web-based tool for the set-up of high-throughput genome-wide mapping panels for the SNP genotyping of mouse mutants. Nucleic Acids Res. **33:** W496–W500.

Klempt, M., B. Rathkolb, E. Fuchs, M. Hrabe de Angelis, E. Wolf *et al.*, 2006 Genotype-specific environmental impact on the variance of blood values in inbred and F1 hybrid mice. Mamm. Genome **17:** 93–102.

Kluppel, M., and J. L. Wrana, 2005 Turning it up a Notch: cross-talk between TGF beta and Notch signaling. BioEssays **27:** 115–118.

Kojro, E., G. Gimpl, S. Lammich, W. Marz and F. Fahrenholz, 2001 Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the alpha-secretase ADAM 10. Proc. Natl. Acad. Sci. USA **98:** 5815–5820.

Lardelli, M., J. Dahlstrand and U. Lendahl, 1994 The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mech. Dev. **46:** 123–136.

LaVoie, M. J., and D. J. Selkoe, 2003 The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J. Biol. Chem. **278:** 34427–34437.

Lehar, S. M., J. Dooley, A. G. Farr and M. J. Bevan, 2005 Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. Blood **105:** 1440–1447.

Li, L., I. D. Krantz, Y. Deng, A. Genin, A. B. Banta *et al.*, 1997 Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat. Genet. **16:** 243–251.

Lindsell, C. E., C. J. Shawber, J. Boulter and G. Weinmaster, 1995 Jagged: a mammalian ligand that activates Notch1. Cell **80:** 909–917.

Marschall, S., and M. Hrabe de Angelis, 1999 Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet. **15:** 128–131.

Martinez Arias, A., V. Zecchini and K. Brennan, 2002 CSL-independent Notch signalling: A checkpoint in cell fate decisions during development? Curr. Opin. Genet. Dev. **12:** 524–533.

McCright, B., 2003 Notch signaling in kidney development. Curr. Opin. Nephrol. Hypertens. **12:** 5–10.

Miyamoto, A., R. Lau, P. W. Hein, J. M. Shipley and G. Weinmaster, 2006 Microfibrillar proteins MAGP-1 and MAGP-2 induce Notch1 extracellular domain dissociation and receptor activation. J. Biol. Chem. **281:** 10089–10097.

Murcia, C. L., N. A. Bilovocky and K. Herrup, 2004 Dissecting complex genetic interactions that influence the Engrailed-1 limb phenotype. Mamm. Genome **15:** 352–360.

Nobta, M., T. Tsukazaki, Y. Shibata, C. Xin, T. Moriishi *et al.*, 2005 Critical regulation of bone morphogenetic protein-induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling. J. Biol. Chem. **280:** 15842–15848.

Nolan, P. M., J. Peters, M. Strivens, D. Rogers, J. Hagan *et al.*, 2000 A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat. Genet. **25:** 440–443.

Oda, T., A. G. Elkahloun, B. L. Pike, K. Okajima, I. D. Krantz *et al.*, 1997 Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat. Genet. **16:** 235–242.

Parks, A. L., K. M. Klueg, J. R. Stout and M. A. Muskavitch, 2000 Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development **127:** 1373–1385.

Pfister, S., G. K. Przemeck, J. K. Gerber, J. Beckers, J. Adamski *et al.*, 2003 Interaction of the MAGUK family member Acvrinp1 and the cytoplasmic domain of the Notch ligand Delta1. J. Mol. Biol. **333:** 229–235.

Przemeck, G. K., U. Heinzmann, J. Beckers and M. Hrabe de Angelis, 2003 Node and midline defects are associated with left-right development in Delta1 mutant embryos. Development **130:** 3–13.

Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk *et al.*, 1999 Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity **10:** 547–558.

Radtke, F., A. Wilson, S. J. Mancini and H. R. MacDonald, 2004 Notch regulation of lymphocyte development and function. Nat. Immunol. **5:** 247–253.

Radtke, F., A. Wilson and H. R. MacDonald, 2005 Notch signaling in hematopoiesis and lymphopoiesis: lessons from Drosophila. BioEssays **27:** 1117–1128.

Shawber, C., J. Boulter, C. E. Lindsell and G. Weinmaster, 1996 Jagged2: a serrate-like gene expressed during rat embryogenesis. Dev. Biol. **180:** 370–376.

Shutter, J. R., S. Scully, W. Fan, W. G. Richards, J. Kitajewski *et al.*, 2000 Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev. **14:** 1313–1318.

Simpson, E. M., C. C. Linder, E. E. Sargent, M. T. Davisson, L. E. Mobraaten *et al.*, 1997 Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat. Genet. **16:** 19–27.

Soewarto, D., V. Blanquet and M. Hrabe de Angelis, 2003 Random ENU mutagenesis. Methods Mol. Biol. **209:** 249–266.

Speca, D. J., N. Rabbee, D. Chihara, T. P. Speed and A. S. Peterson, 2006 A genetic screen for behavioral mutations that perturb dopaminergic homeostasis in mice. Genes Brain Behav. **5:** 19–28.

Threadgill, D. W., D. Yee, A. Matin, J. H. Nadeau and T. Magnuson, 1997 Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. Mamm. Genome **8:** 390–393.

Tsukumo, S., and K. Yasutomo, 2004 Notch governing mature T cell differentiation. J. Immunol. **173:** 7109–7113.

Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon *et al.*, 1996 Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development **122:** 2251–2259.

Vreugde, S., A. Erven, C. J. Kros, W. Marcotti, H. Fuchs *et al.*, 2002 Beethoven, a mouse model for dominant, progressive hearing loss DFNA36. Nat. Genet. **30:** 257–258.

Watabe-Rudolph, M., N. Schlautmann, V. E. Papaioannou and A. Gossler, 2002 The mouse rib-vertebrae mutation is a hypomorphic Tbx6 allele. Mech. Dev. **119:** 251–256.

Weber, J. S., A. Salinger and M. J. Justice, 2000 Optimal N-ethyl-N-nitrosourea (ENU) doses for inbred mouse strains. Genesis **26:** 230–233.

Weinmaster, G., V. J. Roberts and G. Lemke, 1992 Notch2: a second mammalian Notch gene. Development **116:** 931–941.

White, P. H., D. R. Farkas, E. E. McFadden and D. L. Chapman, 2003 Defective somite patterning in mouse embryos with reduced levels of Tbx6. Development **130:** 1681–1690.

Wilson, A., H. R. MacDonald and F. Radtke, 2001 Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J. Exp. Med. **194:** 1003–1012.

Yasuhiko, Y., S. Haraguchi, S. Kitajima, Y. Takahashi, J. Kanno *et al.*, 2006 Tbx6-mediated Notch signaling controls somite-specific Mesp2 expression. Proc. Natl. Acad. Sci. USA **103:** 3651–3656.

Communicating editor: T. R. Magnuson