Haploinsufficiency of the murine polycomb gene Suz12 results in diverse malformations of the brain and neural tube

Xavier Miró1,*, Xunlei Zhou2, Susann Boretius3, Thomas Michaelis3, Christian Kubisch4,5,6, Gonzalo Alvarez-Bolado2 and Peter Gruss1

SUMMARY
Polycomb proteins are epigenetic regulators of gene expression. Human central nervous system (CNS) malformations are congenital defects of the brain and spinal cord. One example of a human CNS malformation is Chiari malformation (CM), which presents as abnormal brainstem growth and cerebellar herniation, sometimes accompanied by spina bifida and cortical defects; it can occur in families. Clinically, CM ranges from an asymptomatic condition to one with incapacitating or lethal symptoms, including neural tube defects and hydrocephalus. However, no genes that are causally involved in any manifestation of CM or similar malformations have been identified. Here, we show that a pathway that involves Zac1 (also known as Plagl1 or Lot1) and controls neuronal proliferation is altered in mice that are heterozygous for the polycomb gene Suz12, resulting in a phenotype that overlaps with some clinical manifestations of the CM spectrum. Suz12 heterozygotes show cerebellar herniation and an enlarged brainstem, accompanied by occipital cortical alterations and spina bifida. Downward displacement of the cerebellum causes hydrocephalus in the most severely impaired cases. Although the involvement of polycomb genes in human disease is starting to be recognized, this is the first demonstration of their role in nervous system malformations. Our work strongly suggests that brain malformations such as CM can result from altered epigenetic regulation of genes involved in cell proliferation in the brain.

RESULTS AND DISCUSSION
Polycomb proteins work in multimeric complexes termed polycomb repressive complexes (PRCs) to regulate the expression of numerous developmental genes that control cell proliferation and stem cell identity, as well as genomic imprinting and X-chromosome inactivation, and are involved, for example, in cancer development (Lee et al., 2006; Sparmann and van Lohuizen 2006; Schuettengruber et al., 2007). Suz12, Eed and Ezh2 are polycomb proteins that combine in PRC2 to silence genes (Cao et al., 2002). In the mouse, Suz12 expression starts with a peak during gastrulation [embryonic day (E)5.5 to E8.5]. This early expression is essential for survival, since Suz12 knockout mice die at gastrulation (as do Eed–/– and Ezh2–/– embryos) (Faust et al., 1998; O’Carroll et al., 2001; Pasini et al., 2004). We detected a second period of intense expression in several organs, including the brain, where expression continues unabated through development and into the adult (Fig. 1A,B). At midgestation, Suz12 was expressed in a posterior-to-anterior gradient in the primordium of the cerebral cortex, as well as in the tectum, hindbrain, eye primordium, somites, first branchial arch, nasal cavity, kidney, lung, liver and pancreas (Fig. 1C–K,O,P). Later, Suz12 transcripts appeared in the cerebellum and hippocampus (Fig. 1L–N). We also found transcripts of Ezh2 and Eed colocalizing with Suz12 in the developing cortex, tectum, nasal epithelia, liver and kidney (Fig. 1Q,R), suggesting a role for PRC2 in the development of these organs.

The role of the late peak of expression has not yet been addressed. However, Drosophila mutants that are heterozygous for Suz12 show major morphological alterations, including homeotic transformations (Birve et al., 2001). Therefore, in order to investigate the role of Suz12 in mouse development beyond gastrulation, we decided to generate a Suz12-deficient mouse line (Fig. 2A,B). The homozygous knockout animals showed an early lethal phenotype, as reported previously (Pasini et al., 2004). The heterozygotes, however, survived and reproduced, although they showed a range of central nervous system (CNS) abnormalities, which were not gender related (Fig. 2C–D’). The consistent increases and decreases in size of specific brain regions were particularly intriguing. In contrast to the anterior cortex (A-CTX) and other normal-sized telencephalic regions, such as the caudate-putamen and hippocampus, the posterior cortex (P-CTX) was smaller in Suz12+/− mice than in wild-type animals (cortical heterometry) (Fig. 2C–G; supplementary material Fig. S1). This cortical heterometry correlated well with the caudo-rostral gradient of Suz12 expression (Fig. 1D,E,P).

We then examined the expression of two marker genes that are expressed specifically in the P-CTX during development, Id2 and COUP-TF1 (also known as Nr2f1) (Armentano et al., 2006; Lasorella et al., 2006). Both of these genes showed shortened domains of expression in the heterozygous mutants, indicating that the cortical reduction in size affects specifically the P-CTX (Fig. 2H–K). By contrast, there was a consistent increase in the size of the midbrain tectum of heterozygous animals, where both the superior...
and inferior colliculi (SC and IC, respectively) were enlarged significantly (Fig. 2E-G; supplementary material Fig. S1). These phenotypical traits co-segregated so that the individuals with the largest reduction in the size of the P-CTX showed, at the same time, the largest increase in IC and SC surface area. Over 60% of the mutants measured had values that were outside the wild-type range (supplementary material Fig. S1). The overall changes in the P-CTX, IC and SC were statistically significant (Fig. 2G).

The enlargement of the IC and SC produced a ‘beaked tectum’ (Fig. 3A,B), which, in the most pronounced cases, caused dramatic cerebellar herniation through the foramen magnum and a secondary hydrocephalus (Fig. 3C,D). Magnetic resonance imaging (MRI) of living *Suz12* heterozygotes (Fig. 3E-H) showed herniation of the cerebellar paraflocculus (corresponding to the human cerebellar tonsils) into the foramen magnum (yellow arrowhead in Fig. 3F,G), with a crowded posterior fossa (white arrowheads in Fig. 3F,G).

Next, we asked what mechanism could produce the alterations in size that we found in the *Suz12* heterozygote brain. The decrease in cortex size or increase in tectum size could be attributed to a dysregulation of cell proliferation. Indeed, bromodeoxyuridine (BrdU)-labeling of brains in heterozygous animals showed strongly reduced proliferation in the P-CTX (Fig. 3I) (apoptosis, as detected by TUNEL, was unchanged; data not shown) and increased proliferation in the tectum (Fig. 3I). Since the role of the PRC is to maintain transcriptional repression, the defects observed in *Suz12*...
heterozygous mice could have resulted from the inappropriate expression of genes involved in neural stem cell proliferation. Some targets of SUZ12 in human embryonic stem cells have recently been mapped by chromatin immunoprecipitation on a genome-wide scale (Lee et al., 2006). One of those targets, ZAC (also known as PLAGL1 or LOT1), encodes a transcription factor that regulates apoptosis and cell cycle arrest in the neuroepithelium (Varrault et al., 2006). Intriguingly, embryonic and postnatal expression of the murine ortholog of ZAC, Zac1, colocalized strongly with that of Suz12 (compare Fig. 3J with Fig. 1P) (Valente and Auladell, 2001). To verify this point, we labeled Suz12 and Zac1 with specific antibodies on sections of E14.5 wild-type brain. The results show
that both proteins are colocinated in the cortical neuroepithelium (ctx in Fig. 3K). The hem region lacks the Suz12 protein (Fig. 3K), in agreement with our mRNA data (Fig. 1E).

Zac1 was upregulated in the Suz12 heterozygous cortex (by real-time PCR and by ISH) (Fig. 3L-N) and tectum (Fig. 3O,P). Cultured fibroblasts from heterozygous embryos also showed Zac1 upregulation (Fig. 3N). To verify a link between the downregulation of Suz12 and the increase in Zac1 expression, we used small interfering RNA (siRNA) to knockdown SUZ12 in a cell line that is commonly used for silencing analysis of the polycomb genes (Aoto et al., 2008), the human osteosarcoma U2Os line (U2Os-Suz12 RNAi cells). Knocking down SUZ12 in these cells resulted in upregulation of ZAC at the protein and mRNA levels (Fig. 3Q). Zac1 exerts its effects by inducing the expression of Pac1 (also known as Adcyap1rl) (Rodriguez-Henche et al., 2002), a receptor present in the neuroepithelium. Injection of the Pac1 ligand pituitary adenylate cyclase-activating polypeptide (PACAP) into the developing rat cortex decreases proliferation (without increasing apoptosis) and enhances the phosphorylation of the cAMP-responsive element-binding protein (CREB) (Suh et al., 2001). In addition, real-time PCR revealed increasing expression of Zac1 in Suz12+/− E13.5 embryonic fibroblasts (MEF) (N). (Q) SUZ12 knockdown in human osteosarcoma U2Os cells resulted in upregulation of ZAC. Transfection with specific siRNA dramatically reduced SUZ12 expression, as confirmed by western blotting and real-time PCR (Q), resulting in upregulation of ZAC at both the protein and mRNA levels (Q). (R,S) Detection of P-Creb by immunohistochemistry at E14.5 revealed an increase in the Suz12+/− P-CTX (arrowheads). The values in I represent the mean±s.d. of measurements on three brains; the values in N,Q represent the mean±s.d. of three experiments. Bars, 500 μm (A-D,F,G); 1 mm (E,H,J); 100 μm (K,R,S); 250 μm (L,M,O,P).
code controls the orderly differentiation of vertebrae (Kessel and Gruss, 1990). The most seriously affected of our heterozygous mice showed a protuberated back (white arrowhead in Fig. 4A) and varying degrees of spina bifida (Fig. 4E-G). These mutants subsequently developed paraplegia (yellow arrowhead in Fig. 4A). Suz12 is indeed expressed in the early neural tube (Fig. 4B-D). Diastematomyelia, a vertical splitting of the spinal cord by a bony, fibrous or cartilaginous septum (Parmar et al., 2003), was also observed in lumbar L3-L4 vertebrae (Fig. 4H), the development of which is controlled by Hoxd9 (Burke et al., 1995). Consistently, it has been shown that SUZ12 binds to the HOXD9 promoter region in human embryonic stem cells (Lee et al., 2006). Finally, some of the Suz12 heterozygotes showed hippocampal morphological alterations (Fig. 4I,J), as well as partial agenesis of the corpus callosum (arrows in Fig. 4K,L). We also observed a loss of gray matter in a gradient matching the posterior-to-anterior gradient of Suz12 expression (see above), together with layer disorganization in the P-CTX (Fig. 4K,L).

The morphological CNS alterations show incomplete penetrance and variable expressivity in the heterozygous mice, and are also found in varying degrees of severity in human patients with neural tube defects and congenital brain malformations (Adeloye, 1976). These severely disabling and, as a group, rather common pathologies represent a continuum of disorders with multifactorial etiology. One of the sub-phenotypes is Chiari malformation (CM), which typically shows herniation of the cerebellar tonsils through the foramen magnum with secondary hydrocephalus. Additional symptoms may include diastematomyelia (Parmar et al., 2003); secondary paralysis of the legs (Stevenson, 2004); abnormal gyral pattern and dysplasia in occipital regions (Stevenson, 2004); and partial agenesis of the corpus callosum. Thus, this syndrome shows partial overlap with symptoms in some of the haploinsufficient Suz12 mice, making a causative role of SUZ12 in human CNS malformations an attractive hypothesis. However, it has to be noted that none of these clinically defined disease entities follows a monogenic mode of inheritance in humans, making it difficult to obtain definite proof that SUZ12 or other genes of the polycomb complex have a causative genetic involvement in disease pathophysiology. Moreover, the Chiari-like phenotype in our mice seems to be the result of the gross enlargement of the tectum, which has not been described as a characteristic primary alteration in CM or other clinically defined human CNS malformation syndromes, leaving the comparability of phenotypes unclear. Nevertheless, a direct or indirect involvement of polycomb-mediated epigenetic changes in CNS malformations still seems attractive, particularly because of the proven association between neural tube defects and folate-dependent methylation pathways (see overview in Kibar et al., 2007).

Additional evidence supporting a role for SUZ12 in human CNS malformations may come from clinical and genetic studies on neurofibromatosis type 1 (NF1), an autosomal dominant phakomatosis affecting 1 in 3500 individuals, which is caused by a heterozygous mutation in the tumor suppressor gene NF1 (Venturin et al., 2004). In addition to the characteristic tumor predisposition and cutaneous symptoms, some NF1 patients do also show severe mental retardation and brain malformations, including disturbances of cortical development (Balestri et al., 2003). The majority of NF1 cases are caused by point mutations in the NF1 gene. However, 5–10% of cases show a larger genomic deletion (Venturin et al., 2004), comprising the SUZ12 and around 12 additional genes, typically resulting in a more severe phenotype. This aggravated form of the disease is probably because of the haploinsufficiency of one or more of the additionally deleted genes, including SUZ12. Moreover, some NF1 patients have been found to show a CM (Tubbs et al., 2004), although a possible link to the type of underlying mutation has not been investigated in this study. By contrast, there is a small study that describes five independent NF1 patients with microdeletions, all of whom show severe developmental impairment and three of whom have structural brain anomalies (including one patient with a CM and hydrocephalus, and another with dysgenesis of the corpus callosum) (Korf et al., 1999). These studies would be compatible with the hypothesis that haploinsufficiency of SUZ12 may be involved in the development of the more severe phenotype of NF1 microdeletion patients and possibly in the development of diverse brain malformations or, more generally, brain malfunction.

Epigenetic alterations are, on the one hand, essential for proper development but, on the other hand and in the case of dysregulation, they are increasingly being linked to a variety of human diseases (Feinberg, 2007). For example, the involvement of alterations in polycomb silencing in the pathogenesis of cancer is an active area of research (Tan et al., 2007). Moreover, our results are the first to indicate that a genetically induced alteration of epigenetic transcriptional control mechanisms can underlie

![Fig. 4. Spina bifida, diastematomyelia and callosal alterations in Suz12+/− mice.](image-url)
Suz12 and Zac1 in mouse CNS malformation

TRANSLATIONAL IMPACT

Clinical issue
Brain and neural tube defects are among the most common human congenital malformations. One prominent example is the Chiari malformation (CM), a developmental defect characterized by abnormal growth of the brainstem and cerebellar herniation. AMs can be asymptomatic or can cause a wide range of clinical conditions. Malformations may, for instance, cause severe neural tube defects and hydrocephalus, in turn leading to incapacitating or lethal conditions. AMs and most other congenital brain defects have a pronounced genetic basis and a complex inheritance pattern. The genetic causes of these defects, and the molecular interplay between known environmental risk factors (for example, folic acid deficiency) and susceptibility genes are currently ill defined.

Results
In this study, the authors identify two genetic factors that cause Chiari-like malformations in mice. One of these genes, Suz12, is a member of a polycomb complex, a group of proteins that act as epigenetic regulators of gene expression. In comparison to normal, wild-type mice, which have two copies of Suz12, mice with only one copy of Suz12 have brain and neural tube defects. Some of the Suz12 heterozygotes show cerebellar herniation and an enlarged brainstem accompanied by occipital cortical alterations and spina bifida, almost perfectly resembling CM as seen in some human patients. The most impaired mice have downward displacement of the cerebellum, which causes hydrocephalus. The authors also demonstrate that Zac1 (also known as Plag1 or Lott1), a regulator of neuronal proliferation, is part of the same molecular pathway affected by Suz12 deficiency.

Implications and future directions
Polycomb proteins are starting to be recognized as having a key role in human disease; this study demonstrates that polycomb protein complexes can play a role in nervous system malformations as well. This work strongly suggests that developmental brain abnormalities such as CMs can result from altered epigenetic regulation of genes involved in cell proliferation in the brain.

doi:10.1242/dmm.003665

ISH probes
IMAGE consortium CloneID (Lennon et al., 1996) and GenBank Accession ID for ISH probes: Suz12, IRAKp961B054Q2 (BC064461, nucleotides 3163-4448); Ezh2, IRAKp9610810Q2 (BC016391, 1576-2595). For Zac1, Eed, LacZ and COLIP-TFI, we amplified probes from E14.5 mouse brain cDNA, covering nucleotides: 1838-2636 of AK087432 (Zac1), 529-1531 of AK077664 (Eed), 324-897 of U89671 (LacZ), and 621-1413 of U07625 (COLIP-TFI). For Id2, RIKEN clone 28104640G09 was used (Kawai et al., 2001) (http://genome.gsc.riken.go.jp/).

MRI
T2-weighted images [2D fast spin-echo (FSE), repetition time (TR)=7 seconds, effective echo time (TE)=42.7 milliseconds, 8 echos, 50 contiguous slices] were obtained in sagittal and axial planes, at a magnetic field strength of 9.4 Tesla (Bruker Biospin GmbH, Germany), at a spatial resolution of 100×100×250 μm³.

Other methods
Brain areas were measured with Scion Image software (Scion Corporation). The real-time PCR primers for Suz12 were 5′-CTCTCCCCTGCAAACGAG-3′ and 5′-CCCCCTGACAC-TATCTG-3′. The RNAi SUZ12-specific sequence was as described (Pasini et al., 2004). For statistics, Student’s t-test was used; P<0.05 was considered significant.

ACKNOWLEDGEMENTS
This work was supported by the Max Planck Society.

COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
X.M., G.A.-B. and P.G. conceived and designed the experiments; X.M. performed the MRI assays; X.M., G.A.-B. and C.K. analyzed the data; X.M., G.A.-B. and C.K. wrote the paper.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl?doi:10.1242/dmm.001602/-/DC1

Received 19 September 2008; Accepted 20 March 2009.

REFERENCES

METHODS
Suz12 mutant mice
Using a genomic clone containing Suz12 from mouse library PAC-RPCI-21 (RPCI711PP78, German Resource Center for Genomics), we targeted exon 12 with lacZ and the PGK-neomycin cassette (Fig. 2A), and then generated Suz12 mutant mice as described previously (Nagy et al., 2003). Animals were handled according to European and German law. PCR primers for wild-type (0.35 kb) and mutant (0.30 kb) alleles: P1, 5′-GCTGGAAGACGATCA-3′; P2, 5′-CCAGGTATCTTGGAG-3′; P3, 5′-TGAGCTTGAGTTACCTG-3′. Northern blot probe: nucleotides 3163-3937 of GenBank ID BC064461 (Suz12).

Antibodies
Primary antibodies: anti-SUZ12 (Upstate), anti-BrdU (Dako), anti-phospho-CREB (Upstate), anti-β-tubulin (Santa Cruz), anti-Zac1 (Santa Cruz). Secondary antibodies: biotinylated universal anti-mouse/rabbit IgG (ABC) and HRP-conjugated anti-rabbit IgG (Amersham).

congenital malformations of the brain and spinal cord, which represent a diverse group of rather common and severely disabling human disorders of the CNS.

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