A genome-wide screen for modifiers of transgene variegation identifies genes with critical roles in development

Alyson Ashe*, Daniel K Morgan**†, Nadia C Whitelaw*†, Timothy J Bruxner*, Nicola K Vickaryous*, Liza L Cox‡, Natalie C Butterfield§, Carol Wicking§, Marnie E Blewitt§, Sarah J Wilkins¥, Gregory J Anderson¥, Timothy C Cox‡ and Emma Whitelaw*

Addresses: *Epigenetics Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Herston, Queensland 4006, Australia. †School of Medicine, University of Queensland, Brisbane, 4001, Australia. ‡Division of Craniofacial Medicine, Department of Pediatrics, University of Washington, Seattle 98195, WA, USA. §Institute for Molecular Bioscience, The University of Queensland, St Lucia, Queensland 4072, Australia. ¥Walter and Eliza Hall Institute, Melbourne, Victoria 3050, Australia. *Iron Metabolism Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Herston, Queensland 4006, Australia.

Correspondence: Emma Whitelaw. Email: emma.whitelaw@qimr.edu.au

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Abstract

Background: Some years ago we established an N-ethyl-N-nitrosourea screen for modifiers of transgene variegation in the mouse and a preliminary description of the first six mutant lines, named MommeD1-D6, has been published. We have reported the underlying genes in three cases: MommeD1 is a mutation in SMC hinge domain containing 1 (Smchd1), a novel modifier of epigenetic gene silencing; MommeD2 is a mutation in DNA methyltransferase 1 (Dnmt1); and MommeD4 is a mutation in Smarca 5 (Snf2h), a known chromatin remodeler. The identification of Dnmt1 and Smarca5 attest to the effectiveness of the screen design.

Results: We have now extended the screen and have identified four new modifiers, MommeD7-D10. Here we show that all ten MommeDs link to unique sites in the genome, that homozygosity for the mutations is associated with severe developmental abnormalities and that heterozygosity results in phenotypic abnormalities and reduced reproductive fitness in some cases. In addition, we have now identified the underlying genes for MommeD5 and MommeD10. MommeD5 is a mutation in Hdac I, which encodes histone deacetylase I, and MommeD10 is a mutation in Bazlβ (also known as Williams syndrome transcription factor), which encodes a transcription factor containing a PHD-type zinc finger and a bromodomain. We show that reduction in the level of Bazlβ in the mouse results in craniofacial features reminiscent of Williams syndrome.

Conclusions: These results demonstrate the importance of dosage-dependent epigenetic reprogramming in the development of the embryo and the power of the screen to provide mouse models to study this process.
Background

Random mutagenesis screens for modifiers of position effect variegation were initiated in *Drosophila* in the 1960s [1,2]. The screens used a fly strain, called *w*, that displays variegated expression of the white (*w*) locus resulting in red and white patches in the eye. Variegation refers to the ‘salt and pepper’ expression of some genes due to the stochastic establishment of an epigenetic state at their promoters. The best characterized example of variegation in mammals is the coat color of mice carrying the *A* <sup>vg</sup> allele [3,4]. In this case there is a correlation between DNA methylation at the promoter and silencing of the gene [5,6]. Alleles of this type provide us with an opportunity to study epigenetic gene silencing in a molecular level in a whole organism.

The extent of the variegation at the *w*<sup>+</sup> locus, that is, the ratio of red to white patches in the eye, was known to be sensitive to strain background, suggesting the existence of genetic modifiers. Offspring of mutagenized flies were screened for changes in the degree of variegation. These screens have been continued to saturation and the results suggest that there are between 100 and 150 such genes [7,8]. Approximately one-third of these have been identified and, as expected, most turn out to play critical roles in epigenetic gene silencing [1,9]. These include genes encoding proteins involved in heterochromatin formation, for example, HP1 and histone methyltransferases [8].

Recently, we established a similar screen in the mouse using a transgenic line that expresses green fluorescent protein (GFP) in a variegated manner in erythrocytes [10]. We anticipated that the screen would produce mutant lines that would help clarify the role of epigenetic gene silencing in mammals. Offspring of N-ethyl-N-nitrosourea (ENU) treated males were screened for changes in the percentage of erythrocytes expressing GFP (measured by flow cytometry). In those individuals in which the percentage of expressing cells was higher or lower than the wild-type mean by more than two standard deviations, heritability was tested. A preliminary description of the first six heritable mutations, which we refer to as *Modifiers of murine metastable epialleles* or *Mommes*, following the screening of 608 G1 offspring, has been published [10].

We reported that all six were dosage-effect genes and five of the six were homozygous lethal, with loss of homozygotes apparent at weaning, but no knowledge of when death occurred. At the time of publication in 2005, none of the underlying genes had been identified. Since then we have identified the underlying mutation in three cases, *MommeD1*, *MommeD2* and *MommeD4*. *MommeD1* is a mutation in SMC hinge domain containing 1 (*SmcH1*), encoding a previously uncharacterized protein containing a domain normally found in SMC proteins and we have gone on to show that this protein has a critical role in X inactivation [11]. *MommeD2* is a mutation in DNA methyltransferase 1 (*Dnmt1*), encoding a DNA methyltransferase, and *MommeD4* is a mutation in *Smarca5*, encoding Snf2h, a chromatin-remodeling enzyme [12]. The finding of *Dnmt1* and *Smarca5*, both known to be involved in epigenetic reprogramming, validates the screen. Here we report an update of the screen, adding four new *MommeDs*, identifying the underlying point mutation in two more cases, and further characterizing the phenotypes associated with hetero- and homozygosity.

Results and discussion

Integration site of the GFP transgene

We have previously reported that the GFP transgene used in this screen has integrated as an approximately 11 copy array on chromosome 1 [10]. We were keen to further characterize the integration site. PCR using primers specific to the 3' end of the transgene construct in combination with degenerate random tagging primers (genome walking) revealed that the transgene had integrated into chromosome 1 at 47,747,486 bp (UCSC web browser, July 2007 assembly). This site of integration is neither centromeric nor telomeric, and so presumably the silencing is related to the multicopy nature of the transgene array [13,14]. The integration site does not disrupt any annotated genes, and is approximately 1 Mb from the closest annotated transcript.

The identification of *MommeD7-D10*

We have now screened an additional 400 G1 offspring and recovered four more *Mommes*, *MommeD7-D10* (Figure 1, Table 1). The fluorescence activated cell sorting (FACS)-based screening is carried out on a drop of blood taken at weaning, using a gate set to exclude 99.9% of autofluorescing cells. Under these conditions, wild-type mice homozygous for the transgene express GFP in 55% of erythrocytes. *MommeD7* is a suppressor of variegation, that is, the percentage of cells expressing the transgene was significantly higher than it was in wild-type individuals (Table 1). *MommeD8*, *D9* and *D10* are enhancers of variegation, that is, the percentages of expressing cells were significantly lower than they were in wild-type individuals (Table 1). The mean fluorescence level in expressing cells also changed. We have reported previously that as the percentage of expressing cells increases, the mean fluorescence of the expressing cells increases [10]. We presume that since the mice are homozygous for the GFP transgene, this is mainly due to an increase in the proportion of expressing cells with two active GFP alleles. However, in the case of *MommeD7* the level was more than double that seen in the wild-type littermates. We hypothesized that this was likely to be the consequence of an increase in the percentage of reticulocytes in the peripheral blood of this mutant, as mature red cells, with no ability to produce new proteins, would have, on average, less GFP than reticulocytes (see below). In the cases of *MommeD8*, *D9* and *D10* the mean fluorescence levels were slightly lower than that seen in the wild-type littermates, consistent with a presumed reduction in the proportion of cells with two active GFP alleles.
For each MommeD, the heritability of the mutation has been tested and confirmed over at least 5 generations by using at least 30 litters. During the breeding of each mutant line, the expression profiles remained constant, consistent with the idea that we were following a single mutation in each case. The frequency with which we found these mutations, 1 in 100 G1 offspring, was similar to our previous results [10].

**Homozygous lethality**

Following heterozygous intercrosses, the proportion of expression types observed in the offspring at weaning was consistent with a semidominant homozygous lethal mutation in the cases of MommeD7 and MommeD9, since only two GFP expression profiles were observed (Figure 1, Tables 1 and 2) and there was a significant litter size reduction in both cases (Figure 2). In the cases of MommeD8 and MommeD10, three expression profiles were observed, suggesting viability of some homozygotes (Figure 1, Tables 1 and 2) and in the case of MommeD10 this was later confirmed by genotyping for the point mutation. In both cases, fewer individuals with the severe phenotypes were observed than predicted by Mendelian inheritance, suggesting reduced viability of the homozygotes (Table 2). This conclusion is supported by significant litter size reductions in both cases (Figure 2). There is also a suggestion of some heterozygous death in the case of MommeD8 and, to a lesser extent, MommeD10 but this is not statistically significant.

**Homozygous lethality occurs at different stages of development**

Litter size reductions following heterozygous intercrosses have already been reported for MommeD1-D6 at weaning [10], but the timing of the losses has only been reported for MommeD1, D2 and D4. MommeD1 is homozygous lethal in females only, with death occurring around mid-gestation [10,11]. MommeD2 and MommeD4 are homozygous lethal at 8.5 days post-coitus (dpc) and 17.5 dpc, respectively [10,12]. Here we describe the timing of the losses for MommeD3, D5, D6, D7, D8, D9 and D10.

Following intercrosses between MommeD3/+ (genotypes determined by GFP fluorescence and progeny testing), dissections at 14.5 dpc suggested that death of homozygotes had already occurred (Table 3). This was confirmed following a FVB/C57 F1 MommeD3/+ intercross, where embryos could be genotyped using microsatellite markers across the linked interval (Table 3). These data suggest MommeD3-/- mice were dying at or before 14.5 dpc. Similar results were obtained for...
MommeD5 at 14.5 dpc (Table 3). Once the MommeD5 point mutation had been found (see below), these crosses were repeated and dissections were performed at 10.5 dpc. Again, a significantly higher than expected proportion of developmentally delayed embryos were detected (Table 3). These embryos were genotyped and found to be MommeD5−/− in all cases, indicating developmental arrest at around 8-9 dpc.

Results obtained for MommeD6 (genotypes determined by GFP fluorescence and progeny testing) were similar (Table 3), suggesting MommeD6−/− embryos arrest around 8-9 dpc.

Following MommeD7−/− intercrosses (genotypes determined by GFP fluorescence and progeny testing), a small but significant increase in abnormal embryos was detected at 14.5 dpc (Table 3). This increase is not enough to account for all expected MommeD7−/− mice. At 17.5 dpc, approximately one-quarter of the embryos were pale (Table 3, Figure 3a), suggesting a red cell defect in the homozygotes. Homozygous MommeD7 mutants were never seen at weaning (Table 2), and preliminary observations suggest that they die in the first few days after birth. Further analysis of adult heterozygous individuals revealed severe splenomegaly (Figure 3b) and a marked increase in reticulocytes in peripheral blood (Figure 3c).

We hypothesized that this increase in reticulocytes was responsible for the larger than expected increase in the average fluorescence level of the GFP transgene in expressing cells observed in this line (Table 1). We performed FACS analysis on whole blood after staining for reticulocytes with propidium iodide. As expected, MommeD7−/+ mice had a threefold increase in the percentage of reticulocytes compared to MommeD7+/+ mice (Figure 3d), and the percentage of GFP fluorescence in both MommeD7−/+ and MommeD7−/− was higher in reticulocytes than mature red cells (Figure 3e).

Table 2

| Genotype ratios of offspring at weaning following heterozygous intercrosses |
|---------------------------------|------------------|------------------|
|                                 | Observed         | Expected if semidominant, homozygous viable | Expected if semidominant, homozygous lethal |
| MommeD7                         |                  |                               |                   |
| Heterozygote*                   | 65               | 60                            |
| Wild type                       | 25               | 30                            |
| MommeD8                         |                  |                               |                   |
| Homozygote*                     | 14               | 27.5                          |
| Heterozygote*                   | 56               | 55                            |
| Wild type                       | 40               | 27.5                          |
| MommeD9                         |                  |                               |                   |
| Heterozygote*                   | 31               | 37.3                          |
| Wild type                       | 25               | 18.7                          |
| MommeD10                        |                  |                               |                   |
| Homozygote* †                   | 14               | 77.25                         |
| Heterozygote* †                 | 187              | 154.5                         |
| Wild type                       | 108              | 77.25                         |

Offspring were classified as wild type, heterozygous or homozygous based on their GFP expression profiles. p-values: MommeD7, not significant; MommeD8, p < 0.005; MommeD9, not significant; MommeD10, p < 0.0001. *Progeny testing of mice classified based on GFP expression was carried out in a number of cases: 5 for MommeD7−/+, 11 for MommeD8−/−, 16 for MommeD8−/+, 8 for MommeD9−/+, 6 for MommeD10−/−, and 11 for MommeD10−/+. In all cases the genotype was as expected. †Presumed genotype was confirmed by genotyping for 8 MommeD10−/− and 79 MommeD10−/− mice.
### Table 3

Embryo dissections to determine time of death of homozygotes

| Name       | 10.5 dpc | 14.5 dpc | 17.5/18.5 dpc |
|------------|----------|----------|---------------|
|            | Normal   | Abnormal or resorbed | Normal     | Abnormal or resorbed | Normal     | Abnormal or resorbed |
| MommeD3    | 48 (60%) | 32 (40%)\(^a\)       | 41 (65%)  | 26 (35%)           |
| MommeD3 (F1) |          |                      |            |                    |
| MommeD5    | 35 (74%) | 12 (26%)\(^b\) \(^c\) | 42 (72%)  | 16 (28%)\(^b\)    |
| MommeD6    | 38 (63%) | 22 (37%)\(^a\)       | 25 (62.5%)| 15 (37.5%)\(^b\)  |
| MommeD7    | 44 (85%) | 8 (15%)\(^f\)        | 63 (74%)  | 22 (26%)           |
| MommeD8    | 101 (94%)| 7 (6%)               |            |                    |
| MommeD9    | 16 (64%) | 9 (36%)\(^a\)        | 19 (59%)  | 13 (41%)\(^a\)    |
| MommeD10   | 54 (93%) | 4 (7%)               | 86 (88%)\(^f\) | 12 (12%)\(^a\) |
| Control cross | 34 (94%) | 2 (6%)               | 266 (93%)|                    |

\(^a\)The abnormal embryos from which DNA could be extracted (17) were genotyped across the linked interval and all shown to be MommeD3\(^{-/-}\).

\(^b\)These abnormal (very small) embryos were genotyped and shown to be MommeD5\(^{-/-}\).\(^c\)These normal embryos were genotyped and found to have the expected ratio of MommeD10\(^{-/-}\) and MommeD10\(^{-/+}\) embryos.\(^d\)Four of the abnormal embryos were able to be genotyped: two were MommeD10\(^{-/-}\) and two were MommeD10\(^{-/+}\). \(^e\)p < 0.0001; \(^f\)p < 0.05.

Although this is only significant for MommeD7\(^{-/-}\) (p < 0.0005), the trend is there for MommeD7\(^{-/+}\) mice (p = 0.07). This is consistent with the idea that a change in the erythroid cell populations contributes to the dramatic increase in the average fluorescence level of the GFP transgene in MommeD7\(^{-/-}\) mice.

Some MommeD8\(^{-/-}\) mice (classified by their GFP expression profile and progeny testing) were viable at weaning but they were rare (Figure 1, Table 2). Following MommeD8\(^{-/-}\) intercrosses, dissections at 14.5 dpc showed no increase in the number of abnormal or resorbed embryos (Table 3). Litter size at birth was not significantly different from that seen in wild-type litters (data not shown), suggesting that the death of most MommeD8\(^{-/-}\) individuals occurred after birth and before weaning. The only obvious phenotypic abnormality seen in MommeD8\(^{-/-}\) mice that survived to weaning was reduced size. MommeD8 homozygotes were significantly smaller (6.60 g ± 0.25 standard error of the mean (SEM)) than their wild-type (8.54 g ± 0.33 SEM, p < 0.001) and heterozygous (8.65 g ± 0.29 SEM, p < 0.0001) littermates.

Dissections following MommeD9\(^{-/-}\) (determined by GFP fluorescence and progeny testing) intercrosses revealed a pattern similar to that seen for MommeD5 and MommeD6, suggesting MommeD9\(^{-/-}\) embryos arrest before 9.5 dpc (Table 3). In the case of MommeD10 the data suggest that death of homozygotes occurred after birth (Table 3), and preliminary data collected at P7 indicated death in the first week of life (data not shown). Some MommeD10\(^{-/-}\) individuals survived to weaning but they were extremely rare. This was confirmed by genotyping once the point mutation was identified.

So in all ten MommeD mice produced so far, homozygosity for the mutation is associated with embryonic or perinatal lethality (Tables 3 and 4).

### Abnormal phenotypes associated with heterozygosity for MommeD7-D10

Extensive phenotyping of the heterozygous MommeD mutant lines has not been carried out. However, in some cases heterozygous effects were obvious, for example, the haematopoietic defect in MommeD7\(^{-/-}\) mice described above. We have also noticed some litter size reduction during the breeding of these strains. The data for the breeding of MommeD7, D8, D9 and D10 are shown in Figure 4. Following crosses between heterozygous males and wild-type females in the FVB strain, we found significant litter size reductions in the cases of MommeD9 and MommeD10, but not in the cases of MommeD7 and MommeD8. A breakdown of the offspring by sex and genotype revealed that for MommeD9 and MommeD10, the litter size reduction was associated with a deviation from Mendelian patterns of inheritance (p < 0.05 in both cases) and a reduction in the number of mutants (Figure 4). These two cases of transmission ratio distortion have not been investigated further but they do suggest that heterozygosity for the MommeD mutations is associated with some level of disadvantage. There also appears to be a skewed sex ratio in the wild-type offspring of MommeD9 sires, suggesting the phenotype of the father can affect his wild-type offspring. While we have not characterized this in any more detail, the idea of a paternal effect is not new. We have previously published examples of paternal effects resulting from haploinsufficiency of modifiers of epigenetic gene silencing in the mouse [12].
Hematopoietic abnormalities in MommeD7 mice. (a) Examples of phenotypically normal and abnormal (pale) embryos at 17.5 dpc. Abnormal embryos are assumed to be homozygous for MommeD7. Scale bar = 5 mm. (b) Spleen weights from MommeD7+/+ and MommeD7−/− adult mice. Error bars represent SEM. ****p < 0.0001. (c) Blood smears from MommeD7+/+ (left) and MommeD7−/− (right) mice stained for reticulocytes (shown with arrowheads). (d) Representative histograms showing propidium iodide fluorescence in MommeD7+/+ (left) and MommeD7−/− (right) mice. In each case 10,000 reticulocytes were counted. Red blood cells (RBC), reticulocytes (RET) and nucleated cells (NC) are shown. (e) Histogram showing relative levels of GFP fluorescence in red blood cells and reticulocytes. Averages were calculated from three wild-type and three heterozygous mice. **p < 0.005; error bars represent SEM.

Mapping
We have mapped the mutations in all ten cases to relatively small regions of the genome (Table 4). The mapping of MommeD1-D6 has been documented [10]. Here we report the mapping of MommeD7-10. MommeD7 maps to a 0.25 Mb region on chromosome 7 between markers D7Mit220 and rs13479441 (using 134 phenotypically mutant and 135 phenotypically wild-type mice). This region contains 10 genes. MommeD8 maps to a 4 Mb region on chromosome 4 between markers rs6337795 and D4Mit279 (using 234 phenotypically mutant and 177 phenotypically wild-type mice). This region contains 54 genes. MommeD9 maps to a 3 Mb region on chromosome 7 between markers rs31712695 and rs6193818 (using 103 phenotypically mutant and 127 phenotypically wild-type mice). This region contains 80 genes. MommeD10 maps to a 4 Mb region between markers D5Mit165 and rs13478547 on chromosome 5. Twenty-four phenotypically homozgyous and 312 phenotypically non-homozygous (heterozygous and wild-type mice combined) were used (see Materials and methods). These data show that each of the ten MommeD mutations maps to a unique region of the genome.

MommeD5 has a mutation in Histone deacetylase 1
MommeD5 was localized to a 1.42 Mb region on chromosome 4 flanked by the markers rs27486641 and rs27541967 [10] (Table 4). This interval contains 46 genes and Hdac1 was chosen as the best candidate because of its known role in chromatin modification. Sequencing of all exons, including exon/intron boundaries, from two heterozygous and two wild-type individuals revealed a 7 bp deletion (GAAGCCA) in exon 13 in the mutants (Figure 5a). This mutation was subsequently verified in over 100 mice classified as mutants by GFP expression profiling. The chance of a second point mutation occurring in a functional region in a linked interval of this size is extremely small. Using the estimated mutation rate following ENU mutagenesis of 1 in 1.82 Mb [15,16], the probability of such an event can be calculated [15,17]. This website takes into account the amount of coding sequence in a given linked interval. In this case, the probability of a second point mutation occurring in the coding region is p < 0.0005.

The mutation produces a frameshift, resulting in changes to the last 12 amino acids, and an additional 25 amino acids. Protein modeling predictions based on human HDAC8, for which the crystal structure has been solved [18,19], suggest that the carboxyl terminus of Hdac1 is relatively unstructured and so the mutation is unlikely to affect the stability of the protein (J Matthews, personal communication). An antibody that recognizes the carboxyl terminus of Hdac1 showed a 50% reduction of binding in 10.5 dpc MommeD5+/+ embryos, and negligible binding in MommeD5−/− embryos (Figure 5b), confirming that this region of the protein has been altered in the MommeD5 line. An antibody that recognizes the amino terminus of Hdac1 showed that the levels of the protein are not altered between mutant and wild-type mice (Figure 5b). Lysine 476 at the carboxyl terminus has been shown to be sumoylated and important for enzymatic function of the wild-type protein [20] and the absence of this amino acid in the MommeD5 mutant protein is likely to impair function. A knockout of Hdac1 has been reported and the homozgyous embryos died around 9.5 dpc [21], similar to the time of death.
### Table 4

Summary of *MommeD* screen for epigenetic modifiers

| Name          | Suppressor or enhancer of variegation | Mapping (Mb) | Mutated gene, or number of genes in interval | Homozygous lethality |
|---------------|---------------------------------------|--------------|---------------------------------------------|----------------------|
| *MommeD1*     | Suppressor                            | Chr 17 (2.4) | *SmcHD1*<sup>a</sup>                         | Females only E10     |
| *MommeD2*     | Suppressor                            | Chr 9 (1.4)  | *Dnmt1*†                                    | E9                   |
| *MommeD3*     | Suppressor                            | Chr 11 (6)   | 123                                         | E9-E14               |
| *MommeD4*     | Enhancer                              | Chr 8 (2.5)  | *Smarca5*†                                  | E17                  |
| *MommeD5*     | Enhancer                              | Chr 4 (1.4)  | *Hdac1*                                     | E8-E9                |
| *MommeD6*     | Suppressor                            | Chr 14 (2.5) | 32                                          | E8-E9                |
| *MommeD7*     | Suppressor                            | Chr 7 (0.25) | 10                                          | P1-P7                |
| *MommeD8*     | Enhancer                              | Chr 4 (4)    | 54                                          | Most P1-P21          |
| *MommeD9*     | Enhancer                              | Chr 7 (4)    | 80                                          | E9                   |
| *MommeD10*    | Enhancer                              | Chr 5 (4)    | *Baz1b*                                     | Most P1-P21          |

All mapping data are current for Ensembl build 37.1, release 49. <sup>a</sup>Reported in [11]. †Reported in [12].

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**Figure 4**

Genotypes and sex of offspring, and litter size following paternal transmission of *MommeD7-D10*. (a) *MommeD7*. (b) *MommeD8*. (c) *MommeD9*. and (d) *MommeD10* show the numbers of male and female offspring of wild-type (WT; black) and heterozygous (grey) genotype produced following a cross between a male heterozygous *Momme* and a wild-type female. *MommeD9* and *MommeD10* both show a trend towards transmission ratio distortion and a significant reduction in litter size compared to wild-type crosses. n represents the number of litters produced.
A mutation in *Hdac1* correlates with the *MommeD5*-/- phenotype. (a) A 7 bp deletion was detected in exon 13 of *Hdac1*. Representative chromatograms from the wild-type (WT) allele, the mutant allele, and one heterozygote are shown. This deletion alters the reading frame, changing the last 12 amino acids and adding 25 extra amino acids. (b) Whole-cell lysates from six individual 10.5 dpc *MommeD5*/*+* and *MommeD5*/*+* embryos, and six pooled *MommeD5*-/- embryos were probed with antibodies to the *Hdac1* carboxyl terminus (top panel), *Hdac2* (top panel) and *Hdac1* amino terminus (bottom panel). Antibody was used as a loading control in each case. Quantification of the *Hdac1* carboxyl terminus relative to γ-tubulin shows negligible binding in *MommeD5*-/- mice. Quantification of *Hdac2* levels relative to γ-tubulin shows increased *Hdac2* in *MommeD5*-/- and *MommeD5*/*-/- embryos. Quantification of *Hdac1* amino terminus relative to γ-tubulin shows equal levels of *Hdac1* in all mice. A peptide blocking experiment was performed to confirm band identity. A representative western blot is shown. Error bars represent SEM. (c) A representative litter from a *MommeD5*/*+* intercross at 10.5 dpc. *MommeD5*/*+* embryos (bottom right) are always dramatically smaller than *MommeD5*/*+* and *MommeD5*/*+* littermates.

**MommeD10 has a mutation in Baz1b**

*MommeD10* was localized to a 4 Mb region on chromosome 5 flanked by the markers D5Mit165 and rs32250347 (Table 4). Interestingly, this interval encompasses the region syntenic with the Williams Beuren syndrome (WBS) critical region in humans. WBS, also known as Williams syndrome, is an autosomal dominant disorder affecting approximately 1 in 10,000 individuals. The classic presentation of WBS includes a characteristic craniofacial dysmorphology (elfin face), supravalvular aortic stenosis, multiple peripheral pulmonary arterial stenoses, statural deficiency, infantile hypocalcaemia and a distinct cognitive profile with mild mental retardation. The linked interval for *MommeD10* contains 52 genes and Baz1b was chosen as the best candidate because it contains a bromo-domain (a domain commonly associated with chromatin remodelers) and has recently been shown to form at least two chromatin remodeling complexes and associate with replication foci and promoters [23-25]. Sequencing of all exons, including exon/intron boundaries, from two homozygous, one heterozygous and one wild-type individual revealed one point mutation (T → G transversion) in exon 7 in the mutants (Figure 6a). This mutation was subsequently verified in over 100 mice classified as mutants by GFP expression profile. The mutation results in a non-conservative amino acid change, L733R, in a highly conserved region of the protein (Figure 6b). Western blot analysis showed reduced levels of Baz1b protein in both embryonic and adult *MommeD10*/*+* tissue, with *MommeD10*/*+* tissue showing intermediate levels (Figure 6c and data not shown), suggesting that the mutant protein is much less stable than its wild-type counterpart. Quantitative real-time PCR performed on cDNA from 14.5 dpc embryos showed all three genotypes have similar levels of mRNA (Figure 6d).
Effects of MommeD5 and MommeD10 on DNA methylation at the transgene locus

Transgene silencing can be associated with changes in both DNA methylation [26,27] and chromatin accessibility [28]. This particular transgene promoter consists of a GC-rich segment of the human alpha-globin promoter, which we were unable to analyze by bisulfite sequencing because the cloned bisulfite converted fragment was refractory to growth in bacteria. The transgene also contains the HS-40 enhancer, which is known to regulate the locus in humans [29]. We analyzed the methylation state at this region by bisulfite sequencing. As predicted from the variegated nature of the transgene expression, the methylation pattern differed from clone to clone in all cases (data not shown). The percentage of methylated CpGs in the HS-40 element was approximately 55% (averaged across all clones) in spleen of 4-week-old wild-type FVB/NJ mice (Figure 7a). Samples from MommeD5+/+, MommeD10+/+, and MommeD10−/− mice showed similar levels of CpG methylation (52%, 47%, 59% respectively; Figure 7a). Mice heterozygous for a null allele of Dnmt3b, which showed an increase in expression of the GFP transgene from 37 ± 3% in the wild-type mice to 55.5 ± 2.5% in the wild-type C57BL/6J mouse strain (60%; Figure 7b) compared to that seen in the wild-type C57BL/6J mouse strain (31%; Figure 7b) compared to that seen in the wild-type C57BL/6J mouse strain (31%; Figure 7b). These data suggest that MommeD5 and MommeD10 mutants alter the expression of the transgene by changing the chromatin state rather than by altering DNA methylation levels. This is consistent with the fact that both genes encode proteins involved in histone modification and chromatin remodeling [21,23-25,30-33]. Modifiers identified in this screen include DNA methyltransferases, chromatin remodelers and genes involved in histone modification, all of which have wide-ranging effects in the genome, making it difficult to unravel direct and indirect effects at any particular locus.

Craniofacial analysis of MommeD10 mice

Surviving MommeD10 homozygotes were significantly smaller than littermates at weaning (Student’s t-test, p < 0.0001; Figure 8a). A similar size differential was evident in utero at 18.5dpc (Student’s t-test, p < 0.01), indicating that this is not simply due to poor post-natal feeding (Figure 8a). MommeD10 homozygotes also appeared to have widened, bulbous foreheads and shortened snouts (Figure 8b). To examine the craniofacial phenotype more accurately, three heads from 4-week-old male mice of each genotype (MommeD10+/+, MommeD10+/− and MommeD10−/−) were subjected to micro-computed tomography. Heads from one 4-week-old female of each genotype were also examined at this level. They followed the same trend as males. Visual inspection of the three-dimensional reconstructions confirmed the original observation that homozygote’s skulls were more bulbous and showed a flattening of the nasal bone and upward curvature of the nasal tip (Figure 8c).

Twenty cranial landmarks and nine mandibular landmarks were located on each skull using approximately 70 micron resolution datasets and inter-landmark measurements were compared (Figure 8d and Additional data file 1). Statistical analyses were carried out using the data collected from males only. Homozygote skulls were significantly different to wild
type (Student’s $t$-test, length:height ratio, $p < 0.01$; width:height ratio, $p < 0.01$; length:width ratio, $p < 0.05$), confirming the bulbous appearance of the skulls on the reconstructed images. Much of this difference could be attributed to reduction of the parietal and nasal bones (both $> 12.5\%$ shorter in homozygotes compared to an overall mean length and width reduction of approximately 9%). The reduced parietal bone length and the reduction and upward angulation of the nasal bones in these mice (Figure 8c, d) are reminiscent of the decrease in the volume of the parieto-occipital region and the appearance of the nose in WBS patients [34,35]. Heterozygotes also had a decreased cranium width:height ratio (Student’s $t$-test, $p < 0.05$) and decreased length:height ratio (Student’s $t$-test, $p < 0.05$) compared to wild-type skulls. Of note, heterozygotes showed a reduction in palatine bone width of similar magnitude to that seen in homozygotes, suggesting a greater sensitivity of some parts of the skull to decreased Baz1b protein levels. Measurements of the lower jaw revealed relative mandibular hypoplasia in homozygotes that was most pronounced in the posterior region (approximately 20% reduction), encompassing the condyle, angle and coronoid processes (Figure 8d and Additional data file 1). The posterior aspects of the mandibles of heterozygotes were also reduced in size when compared to wild-type mandibles, albeit to a lesser degree than in the homozygotes.

**Expression of Baz1b during mouse embryogenesis**

It has previously been shown that Baz1b is expressed in the mouse embryo from around 9.5 dpc and whole mount *in situ* at 11.5 dpc showed expression in limb buds, tail and brain [24]. We have gone on to characterize the expression of Baz1b in more detail, and show that at 8.25 dpc Baz1b is expressed in the headfolds, the caudal tail bud region and the presumptive hindbrain in a pattern reminiscent of rhombomere staining (Figure 9a, f). From 9.5 dpc expression is evident in the somites and in the forelimb bud as it emerges from the lateral plate mesoderm (Figure 9b). Diffuse mesenchymal expression in both the forelimb and hindlimb continues until 12.5 dpc when it is restricted to the interdigital mesenchyme (data not shown).

In the facial primordia, Baz1b is expressed in branchial arch 1 as it first emerges (Figure 9g), and continues later in development in both the maxillary and mandibular components of branchial arch 1 and branchial arch 2 (Figure 9c-e, h, i). Expression in the branchial arches is primarily mesenchymal,
MommeD10<sup>−/−</sup> mice are smaller than their littermates and display craniofacial abnormalities. (a) Body weight was measured for 46 MommeD10<sup>+/+</sup>, 102 MommeD10<sup>+/−</sup> and 10 MommeD10<sup>−/−</sup> weaners (3 weeks), and 11 MommeD10<sup>+/+</sup>, 22 MommeD10<sup>+/−</sup> and 5 MommeD10<sup>−/−</sup> embryos (18.5 dpc). Histograms show mean and SEM. (b) Craniofacial abnormalities in adult MommeD10<sup>−/−</sup> mice. MommeD10<sup>−/−</sup> mice display shorter snouts than age and sex-matched wild-type littermates. (c) Three-dimensional reconstruction of skull microCT data from 4-week-old male mice reveals distinct anomalies in homozygous Baz1B mice. Left side: lateral views show the overall size and shape of heterozygous skulls is similar to that of wild-type skulls, whereas skulls of homozygotes were around 20% shorter. Homozygous skulls showed variable anomalies, but consistently had a bulbous appearance, and a short, flattened, or upwardly angulated nasal bone (yellow arrowhead). Slight angulation of the nasal bones was also noted in one heterozygote. Right side: dorsal view of the homozygote skull shown on the left side showing the abnormal shape and more rostral connection of the zygomatic process with the squamosal bone (yellow arrow), skewing of the midline frontal bone suture (black arrow) and minor bilateral anomalies of the frontal/parietal suture (black arrowheads). (d) Twenty cranial landmarks and nine mandibular landmarks (based on those of Richtsmeier [49]) were located on each of nine skulls and inter-landmark measurements recorded. The mean value of each measurement, including analysis of cranium height:width and cranium length:height ratios, was compared between homozygous, heterozygous and wild-type animals.
and is enriched in the rostral distal margin of the mandible, and the caudal distal margin of branchial arch 2 (Figure 9c-e, h). Baz1b is also expressed in the frontonasal process (Figure 9b) and later in the mesenchyme of both the medial and lateral nasal prominences as they elevate to surround the nasal arch 2; Fl, forelimb; Fnp, frontonasal process; Hb, hindbrain; Hf, headfold; Hi, hindlimb; Lnp, lateral nasal prominence; Md, mandibular prominence; Mnp, medial nasal prominence; Mx, maxillary prominence; R, rhombomere; S, somite; Tb, tailbud.

A possible role for Baz1b in Williams syndrome
Overall, the skull shape in mutant animals is reminiscent of the head shape seen in WBS, including a small upturned nose with flat nasal bridge, micromaxilla (or mandibular hypoplasia), malocclusion, bi-temporal narrowing and prominent forehead [34]. WBS is known to be associated with a hemizygous deletion of approximately 28 genes in humans, but which of these genes are responsible for the craniofacial phenotype remains controversial. People with atypical deletions, and varying degrees of craniofacial abnormalities, implicate both proximal and distal ends of the deletion, suggesting that more than one gene is responsible [36-41]. Tassabehji and colleagues [42] reported craniofacial defects in a transgenic (c-myc; Tgf-α) mouse line that harbored a chromosomal translocation fortuitously disrupting the Gtf2ird1 gene, the human orthologue of which resides in the WBS critical interval [43]. Mice homozygous for this transgene produced little Gtf2ird1 mRNA and exhibited craniofacial dysmorphism, suggesting a role for Gtf2ird1 in the craniofacial phenotype. Mice hemizygous for the transgene were indistinguishable from wild-type animals. Disruption of Gtf2ird1 in this mouse was associated with a 40 kb deletion at the site of integration, the addition of 5-10 tandem copies of a c-myc transgene, and translocation of the entire segment to chromosome 6 [43], providing opportunity for disruption to the expression of other genes, such as Baz1b, in the region. A targeted knockout of Gtf2ird1, produced by others, failed to find craniofacial dysmorphology or dental abnormalities [44]. We checked the sequence and expression of Gtf2ird1 in MommeD10 mutants and found no abnormalities (data not shown). The chance of a second point mutation occurring in a coding region in this linked interval is extremely small (p = 0.0008, based on a mutation rate of 1 in 1.82 Mb) [15-17].

Our studies show that the chromatin remodeler Baz1b is expressed strongly in cranial neural crest-derived mesenchyme that drives facial morphogenesis and that homozigosity for a missense mutation in Baz1b produces an array of craniofacial features that are similar to those that characterize the typical WBS face. Significantly, some craniofacial features are also apparent in heterozygotes. These results suggest that reduction in Baz1b protein levels contributes to the elfin facies characteristic of WBS and that WBS is, at least in part, a chromatin-remodeling factor disease.

Conclusion
Extension of the screen has produced four new MommeDs, MommeD7-D10, all of which behave in a semidominant fashion, as do the six previously reported [10]. We have now identified the underlying genes in five of the ten cases, two of which, Hdac1 and Baz1b, we report here. Both are already known to be involved in epigenetic processes, further validating the screen design. In the case of Baz1b this is the first report of a mouse carrying a disrupted allele at this locus and we have shown a role for the protein in craniofacial development.

The screen, designed primarily to identify genes involved in the epigenetic gene silencing of foreign DNA, such as transgenes, has revealed the critical role that such genes play in embryonic and fetal development. It is interesting that at least half of the MommeDs are important during gastrulation. Furthermore, the identification of heterozygous effects sug-
gests that a reduction in the amount of protein product of less than 50% has effects on the health of the mouse. One of the hallmarks of epigenetic gene silencing across all multicellular organisms from plants to humans is the stochastic nature by which they operate [45] and these studies re-emphasize the importance of probabilistic events during embryogenesis. We believe that this screen will provide new tools with which to study these processes.

Materials and methods

Mouse strains

Wild-type inbred C57BL/6J and FVB/NJ mice were purchased from ARC Perth. Procedures were approved by the Animal Ethics Committee of the University of Sydney and the Animal Ethics Committee of the Queensland Institute of Medical Research. The ENU screen was carried out in the FVB/NJ inbred transgenic line as described previously [10]. All MommeD lines were maintained in this strain unless stated otherwise. Most crosses between MommeD individuals were performed on individuals three generations or more removed from the MommeD progenitor. A total of 1,000 G1 offspring were screened at 3 weeks of age, from which ten heritable dominant mutations were identified.

The Dnmt3b null mice were a kind gift from En Li. They have been subsequently backcrossed for more than ten generations to the C57BL/6J strain. GFP fluorescence in Dnmt3b+/+ mice was analyzed in the F1 offspring of crosses between Dnmt3b+/+ and the FVB/NJ transgenic line, and as such each mouse was hemizygous for the transgene.

Flow cytometry

GFP fluorescence in erythrocytes was analyzed by flow cytometry at weaning. A drop of blood was collected in Osmosol buffer (Lab Aids Pty Ltd, Narrabeen, NSW, Australia) and analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) with excitation at 488 and 550 nm. The 488 nm channel predominantly measures GFP fluorescence and the 550 nm channel measures autofluorescence. The data were analyzed using CELL QUEST software with a GFP-positive gate set to exclude 99.9% of wild-type erythrocytes. Mean fluorescence was calculated using cells within the positive gate. Histograms depict only the GFP fluorescence channel.

Genome walking

Genome walking was performed using the APAgene™ GOLD Genome Walking Kit (Bio S&T Inc., Montreal, Quebec, Canada) following the manufacturer’s instructions. Gene specific primers used were (5’-3’): WalkA CCATATTTTCACCATA-CACGACA; WalkB GAGACTTTCTCATCCCCAAAACT; WalkC CCCCCAAAACGTGACCACAA.

Linkage analysis

For MommeD7, D8 and D9, FVB/C57 F1 heterozygous individuals were backcrossed to C57, and linkage analysis performed on their offspring. Phenotype was assigned based on GFP fluorescence profile. A panel of microsatellite markers that differ in size between FVB and C57 were used to localize the mutation to a small area of the genome. Mice wild type for the mutation should only have C57 chromosomes at the linked interval, while mice heterozygous for the mutation should carry both FVB and C57 chromosomes.

Linkage analysis in MommeD10 was carried out using an FVB/C57 F1 MommeD10+/+ intercross to produce 337 F2 individuals. MommeD10+/+ mice were distinguished from their littermates by their dramatically reduced size at weaning and their reduced GFP expression profile. Recombination events allowed the linked region to be localized to a small genomic interval. MommeD10+/+ mice should only carry FVB chromosomes at the MommeD10 linked region, while the remaining mice should be either FVB/C57 or C57/C57.

Reticulocyte counts

Blood smears were made from blood taken from the tail tip of MommeD7+/+ and MommeD7+/+ mice, and stained with New methylene blue. Full blood analyses were done on the automated haematology analyzer Sysmex Xe-2100 (Sysmex Corporation, Woodlands Spectrum, Singapore).

Reticulocyte analysis by FACS

Nucleated cells and reticulocytes were separated from mature erythrocytes based on propidium iodide fluorescence levels. An RNase control was performed and the presumptive reticulocyte cell population could no longer be detected. Mean GFP fluorescence was determined for reticulocyte and mature erythrocyte cell populations. This was done essentially as described in [46]. Three adult MommeD7+/+ and three wild-type littermate controls were used. Approximately 25 µl of whole blood was collected from the tail in cold Osmosol buffer (Lab Aids Pty Ltd). Cells were fixed for 1 h at 4°C in 1% paraformaldehyde in Osmosol and then washed once in cold Osmosol. Cells were then permeabilized by adding 20°C ethanol to a cell pellet whilst vortexing, and incubated with rotation for 2.5 h at 4°C. A 40 µg/ml solution of propidium iodide was added to pelleted cells and the cells were incubated at 37°C for 30 minutes. Analysis was performed on a FACScan (Becton Dickinson). The data were analyzed using CELL QUEST software.

Genotyping assay

Following identification of the MommeD10 point mutation, genotyping was carried out by AciI digestion of a PCR product of exon 7 of Bzzt (primers available on request). The AciI site is created by the MommeD10 point mutation. Following identification of the MommeD5 mutation, genotyping was carried out by PCR amplification across the deleted region, and gel electrophoresis using Ultra low-range agarose (Bio-Rad, Hercules, CA, USA).
Protein analysis

We prepared whole-cell lysates of 10.5 dpc embryos (MommeD5) and 14.5 dpc heads and adult spleen (MommeD10) in 2-3× volume lysis buffer (0.05 M Tris, 7 M urea, 150 mM NaCl). Samples were incubated on ice for 20 minutes, sonicated, centrifuged, and the supernatant collected. Approximately 5 µg (MommeD5) or 10 µg (MommeD10) of protein was separated by SDS-PAGE on a 4-12% gradient gel (Invitrogen, Carlsbad, CA, USA) and was analyzed with antibodies to the Hdac1 amino terminus (sc-6299, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Hdac2 carboxyl terminus (05-100, Millipore, Billerica, MA, USA), Hdac2 (05-814, Millipore), Baz1B (BL2067, Bethyl Laboratories, Montgomery, TX, USA) or γ-tubulin (T5192, Sigma-Aldrich, St Louis, MO, USA). Blots were quantified by normalizing the levels of Hdac1, Hdac2 or Baz1B in each lane to that of γ-tubulin. The normalized levels were then averaged across genotypes and the ratio to wild-type levels calculated. A peptide blocking experiment was carried out for the Hdac1 amino terminus using sc-6299P (Santa Cruz Biotechnology).

RNA preparation and quantitative RT-PCR

Total RNA was extracted from 14.5 dpc embryo heads using TRI reagent (Sigma-Aldrich). cDNA was synthesized from total RNA using SuperScriptII reverse transcriptase (Invitrogen) and random hexamer primers. Quantitative RT-PCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG with primers designed to span exon/intron boundaries (available on request). All reactions were performed in triplicate and normalized to both GAPDH and ribosomal protein; the normalized levels were then averaged across genotypes and the ratio to wild-type levels calculated. A peptide blocking experiment was carried out for the Hdad1 amino terminus using sc-6299P (Santa Cruz Biotechnology).

Bisulfite sequencing of the transgene HS-40 enhancer

Bisulfite sequencing was carried out as described in [48]. Oligonucleotides to the bisulfite converted HS-40 enhancer were as follows (5’-3’): GFPbisF1 AAAATAAAATTTTTGGATTGT-TATTATTATAA; GFPbisF2 ATATTTGTAATTTTAGTTT-GGGAGGTTT; and GFPbisR AATCTCTACTCACAATCTACCACATC. Cycling conditions were as follows: 94°C for 2 minutes for 1 cycle; 94°C for 30 s, 60°C for 30 s, 72°C for 45 s for 35 cycles; and 72°C for 6 minutes for 1 cycle.

Micro-CT analysis

Three heads of 4-week-old male mice of each genotype, and one head of female mice of each genotype (MommeD10+/+, MommeD10+/– and MommeD10−/−) were scanned at 8.7 micron resolution using a SkyScan 1076 micro-computed tomography unit and the data set reduced to approximately 17 microns for three-dimensional reconstruction of the serial slices (CT Analyser software V.1.6.1.1; SkyScan, Kontich, Belgium). Twenty cranial landmarks and nine mandibular landmarks (based on those of Richtsmeier [49]) were located on each of nine skulls and inter-landmark measurements recorded using DataView software (V.1.3.0.0; SkyScan). To verify accuracy of the measurements, any landmarks showing marked differences between genotype groups were re-located on a separate day and the measurement repeated. The mean value of each measurement was compared between homozygotes, heterozygotes and wild-type animals.

RNA probes and whole-mount in situ hybridization

DIG-labeled RNA probes were transcribed from linearized DNA templates and used in whole mount in situ hybridization analysis of wild-type FVB/NJ mouse embryos at a range of gestational stages. The probe was directed to 1.1 kb of the 3’ untranslated region of Baz1B. A sense probe was used in earlier experiments to confirm specificity of the antisense probe. Whole mount in situ hybridization was performed as previously described [50]. Embryo images were captured digitally using an Olympus SZX12 microscope with DP Controller software (Olympus Corporation).

Abbreviations

dpc: days post-coitus; ENU: N-ethyl-N-nitrosourea; FACS: fluorescence activated cell sorting; GFP: green fluorescent protein; Momme: Modifier of murine metastable epialle; SEM: standard error of the mean; WBS: Williams Beuren syndrome.

Authors’ contributions

AA carried out the genetic and molecular studies for MommeD2 and MommeD10, the FACS analysis on whole blood for MommeD7 and co-wrote the manuscript. DKM carried out the genetic and molecular studies for MommeD5. NCW carried out the genetic studies on MommeD7, MommeD8, and MommeD9 and determined the transgene integration site. TJB carried out genetic studies on MommeD6 and initial analysis on MommeD9. NKV performed ENU mutagenesis and carried out genetic studies on MommeD4 and initial analysis on MommeD7. LLC carried out the craniofacial analysis. NCB and CW performed and interpreted the whole mount in situ hybridizations. MEB performed the reticulocyte staining. TCC carried out the craniofacial analysis and interpretation. EW conceived the study, and project design, coordinated the project and together with other co-authors interpreted results and wrote the manuscript. All authors read and approved the final manuscript.

Additional data files

The following additional data file is available with the online version of this paper. Additional data file 1 shows more craniofacial measurements between MommeD10+/+, MommeD10+/– and MommeD10−/− mice.
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