Chapter 4

Generation and verification of SAP102 targeted mice

4.1 Production of SAP102 targeted mice

To engineer a loss-of-function mutation in SAP102 in mice, the constitutive targeting vector generated in the previous chapter (see figure 3.1) was used to target the SAP102 locus in HM-1 mouse embryonic stem cells. These cells originate from 129P2 strain mice, formerly known as 129/OlaHsd (Festing et al., 1999; Ledermann, 2000; Selfridge et al., 1992). Cells electroporated with the linearised vector were cultured with G418 to select those carrying the *neo* selection cassette.

DNAs from G418-resistant colonies were analysed for the desired homologous recombination event with two independent long-range PCR reactions, one using a reverse primer (P2) hybridising to the region deleted by the mutation and the other using a reverse primer (P3) in the selection cassette. The two reactions use a common forward primer (P1) upstream of the 5' homology arm. Figure 4.1a shows the location of these primers in the SAP102 targeted region. Since the ES cells are male and carry only a single X chromosome, homologous recombination of the targeting vector with the endogenous SAP102 locus will convert the cell from wild-type (+/Y) to hemizygous (-/Y). As expected, genomic DNA from wild-type ES cells and most G418-resistant clones produced a 3.85 kb PCR amplification product from primers P1 and P2 but nothing from P1 and P3. DNA from a small number of clones, however, produced a 3.9 kb product from primers P1 and P3 but no 3.85 kb wild-type band. The genotype of the putatively targeted clones was confirmed by southern blot using probes outside the region of homology. Figure 4.1a shows the location of the 5' probe and *Dra*I restriction sites used in the Southern blot. Genomic DNA from wild-type ES cells, adult mouse tail and the G418-resistant but untargeted ES clones produced the expected 12.2 kb wild-type band when digested with *Dra*I and hybridised with the 5' probe. In targeted clones, the probe hybridised only with a 4.8 kb band as predicted by the restriction sites, as shown in figure 4.1c. These results were confirmed using *Nhe*I-digested genomic DNA hybridised with a probe 3' of the targeted region (data not shown).

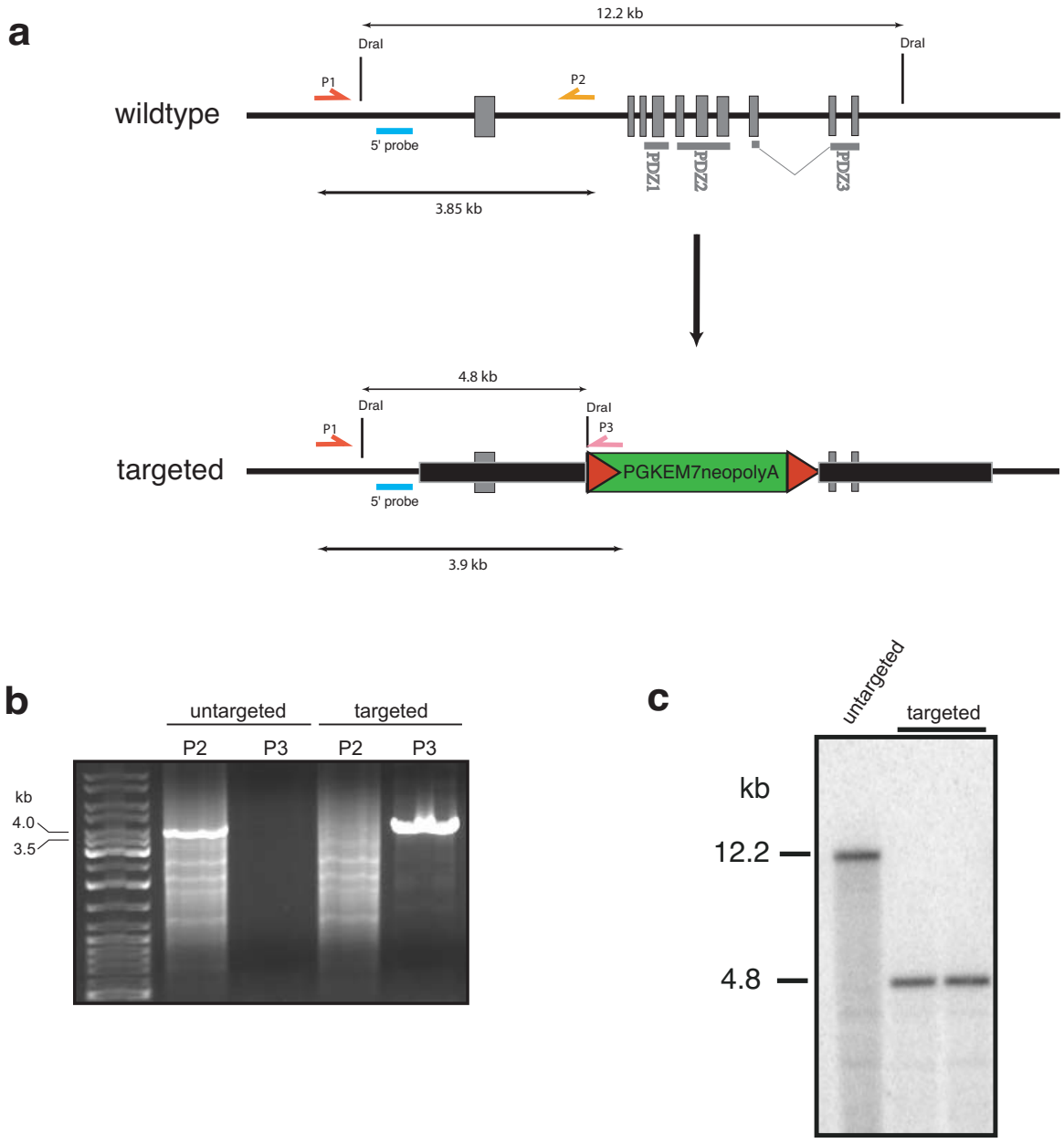

Figure 4.1 Targeting SAP102 in mouse ES cells. (a) The SAP102 deletion encompasses exons 2-8 inclusive, including PDZ domains 1 and 2, and creates a frameshift mutation between exons 1 and 9. PCR primers, restriction sites and the DNA probe used for genotyping are shown. Filled black boxes show the extent of homology arms used for targeting. **(b)** Following electroporation of the targeting construct and antibiotic selection, resistant ES cells are screened by long-range PCR using a forward primer 5' of the targeted region (P1) and two reverse primers, one hybridising to the deleted section of wild-type sequence (P2) and the other hybridising to the selection cassette (P3). Random integration of the targeting construct leaves the SAP102 locus intact so that a PCR reaction using P1 and P2 produces a 3.85 kb band while P1 and P3 generated no product. Conversely, targeted clones produce a 3.9 kb amplification fragment from P1 and P3 but generate no wild-type product. **(c)** The genomic structure of the mutation in targeted clones is confirmed by Southern blot. The 5' DNA probe is radiolabelled and hybridised to DraI-digested genomic DNA. The 12 kb wild-type fragment is reduced to 5 kb in the targeted allele by a DraI site in the selection cassette.

Of 304 G418-resistant colonies analysed, seven were positive for the mutation and two contained both wild-type and targeted DNA, probably the result of two clones, one having undergone homologous recombination and the other random integration of the construct, growing together as one colony in the dish. No unexpected DNA rearrangements were observed in any of the tested clones. These figures give an overall targeting efficiency of approximately 3%.

Two different targeted clones were injected into C57BL6/J mouse blastocysts which were then implanted into pseudopregnant mice. Among the resulting offspring, chimeras carrying cells derived from the targeted (129 strain) ES cells identified by coat colour.

Generation of hemizygous male mice for experimental analyses

Male chimeras from one of the two targeted lines were crossed with wild-type, MF1 strain females. Female (XX) offspring from these crosses inherit an X chromosome from each parent, while male offspring (XY) receive their single X chromosome from their mother and the Y chromosome of their father. Thus, passing of the targeted cells through the male germline produces SAP102 heterozygous (+/-) female and wild-type male (+/Y) offspring.

The heterozygous female offspring were bred with wild-type male offspring from the same crosses to produce hemizygous (-/Y) and wild-type male littermates (see figure 5.1b). Mice were weaned, ear-marked and tail tipped at 3-4 weeks of age and the tail tissue used for genotyping. Genotypes of mice used for further experiments were confirmed by a second PCR assay on tail tissue taken at the end-point of the experiment. All experiments were performed on male littermate pairs. Where females were used as well, they were wild-type and heterozygous littermate pairs. Experimental mice were at least six weeks old unless otherwise indicated and all conclusions are based on results from at least four pairs of mice of the same sex.

4.2 Genotyping of SAP102 targeted mice

Genotypes of targeted mice were initially confirmed with the same Southern blot analyses used to identify positive ES cell clones. DNA from wild-type, heterozygous and hemizygous targeted mice produced the expected patterns of wild-type and targeted bands when digested and hybridised with the 5' (figure 4.2 and b) and 3' (data not shown) probes. Mice inherited the targeted allele in approximately mendelian ratios and no unexpected DNA rearrangements were observed.

Having confirmed the fidelity of the mutation, subsequent mice were genotyped using two short PCR reactions, one amplifying the wild-type allele with a reverse primer (P5) in the deleted region and a forward primer (P4) a short way upstream, and a second reaction amplifying the mutant allele using the same forward primer along with reverse primer P3 in the cassette. The location of these primers is shown in figure 4.2a. Wild-type DNA produced only the 535 bp P4-P5 band, hemizygous DNA produced only the 215 bp P3-P5 band while heterozygous DNA produced both bands, as shown in figure 4.2c.

4.3 SAP102 protein is absent in targeted mice

To examine the effect of the targeted mutation on SAP102 protein production, total protein was extracted from dissected forebrains of adult wild-type and hemizygous male mice and analysed by western blot using an antibody raised against the N-terminal (undisturbed) region of SAP102 (see appendix 1 for a full list of primary antibodies). Figure 4.3a shows a band of approximately 100 kDa is robustly present in wild-type but undetectable in hemizygous mutant forebrains. An additional, non-specific band appears at approximately 60 kDa in both wild-type and mutant extracts, showing equal loading in each lane of the blot. Stripping the blot and re-probing with an antibody against PSD-95 confirmed equal loading (figure 4.3a).

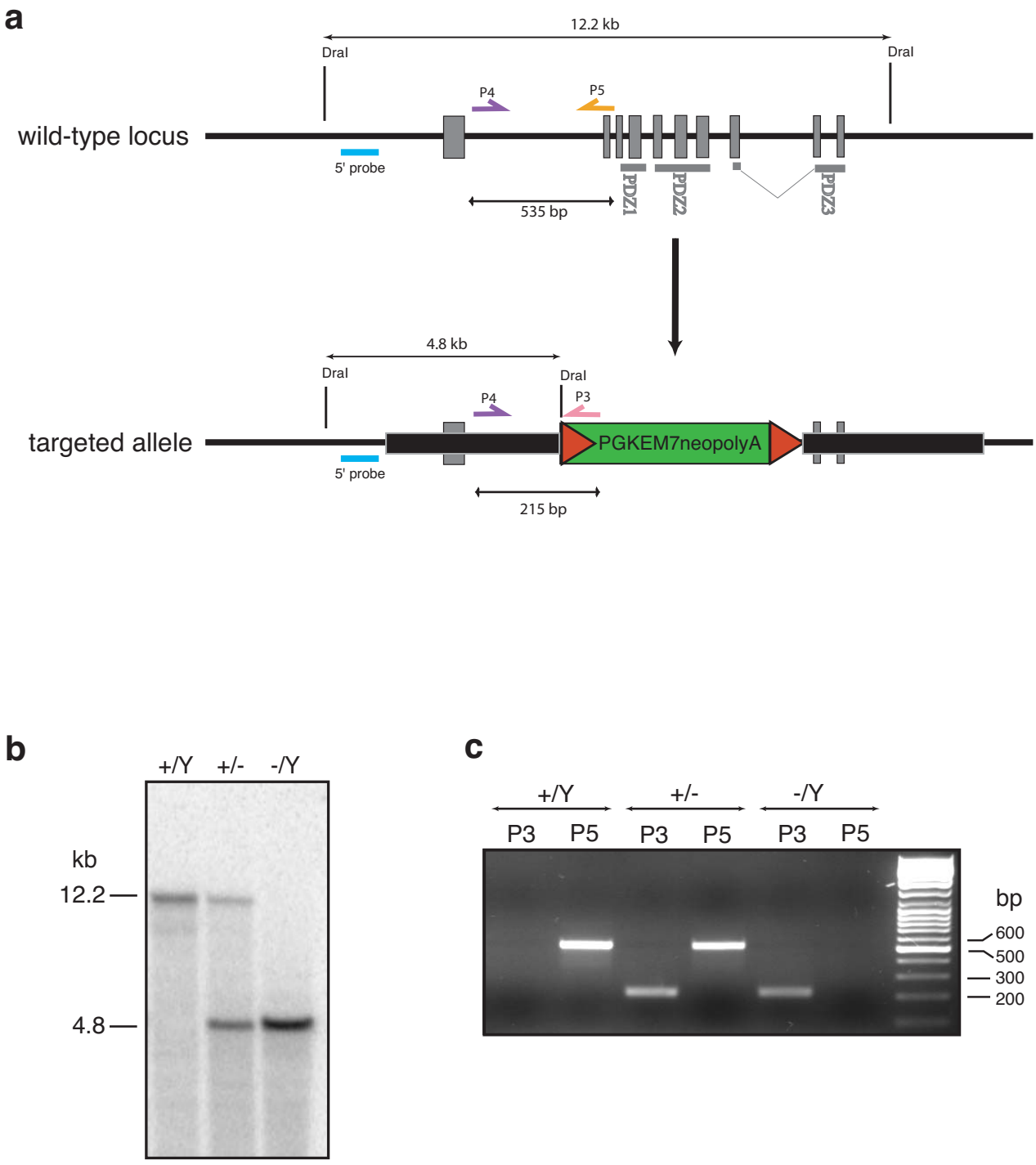

Figure 4.2 Genotyping SAP102 targeted mice. (a) Diagram of the targeted SAP102 genomic locus showing PCR primers, restriction sites and the DNA probe used for genotyping. **(b)** Southern blot confirms the structure of the SAP102 targeted locus, using the same assay as in ES cells (see figure 4.1). DraI-digested DNAs from wild-type (+/Y), heterozygous (+/-) and hemizyous (-/Y) mice produce the expected combinations of wild-type 12.2 kb and mutant 4.8 kb DraI fragments when hybridised with the 5' probe. **(c)** Short-range PCR genotyping assay using a common forward primer, P4, and two reverse primers, P3 and P5, hybridising to the targeted and wild-type alleles respectively. DNA from wild-type, heterozygous and hemizygous mice produce the expected combinations of the 215 bp P4-P3 and 535 bp P4-P5 fragments.

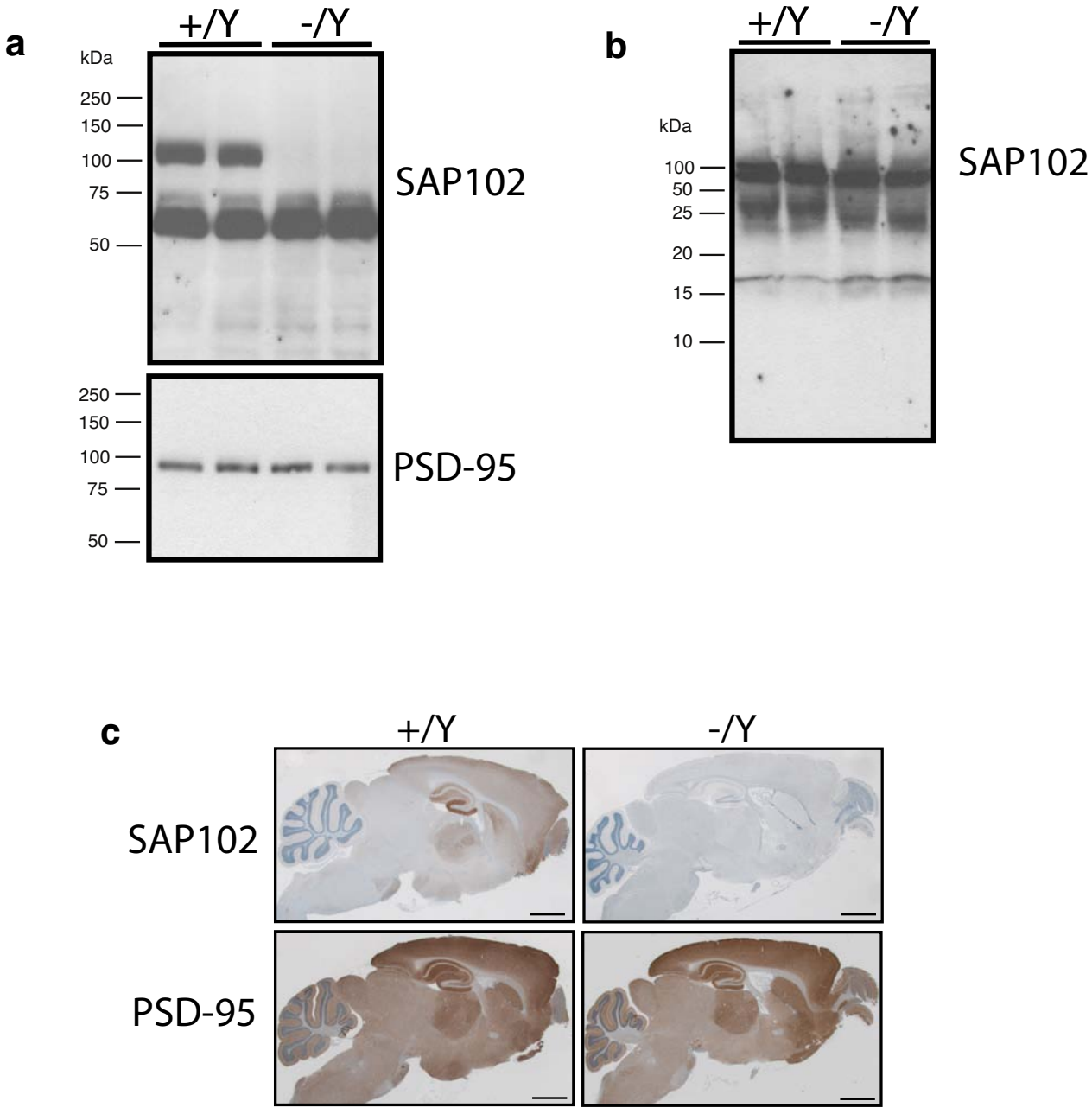

Figure 4.3 SAP102 protein is absent from targeted mice. (a) Western blot analysis of whole forebrain protein extracts shows a strong band at approximately 100 kDa in wild-type mice which is undetectable in hemizgyous mutants. PSD-95 levels are unaffected in the mutants. **(b)** No truncated SAP102 peptide is detectable in forebrain protein extracts from hemizygous mutant mice. The truncated product is expected to be 13 kDa in size. **(c)** Immunohistochemical staining of SAP102 (brown) in parasagittal sections shows the protein is absent from all regions of the brain in hemizygous mice while PSD-95 staining is unaffected. Sections are counterstained with haemotoxylin (blue). For both (a) and (b) antibodies were raised against the N-terminal, unaffected section of SAP102 protein. Scale bars are 2 mm.

The targeted mutation leaves intact the coding sequence for the N-terminal 120 amino acids of SAP102, which could potentially produce a 13 kDa truncated peptide. Such a peptide could not be detected in western blots even when 18 % polyacrylamide gels were loaded with three times the usual amount of protein extract and the blots were exposed for 30 minutes, as shown in figure 4.3b.

Immunohistochemical staining of parasagittal sections from wild-type and hemizygous adult brains was then undertaken to see whether any full-length or truncated protein remained in any part of the brain. For these experiments a different antibody, raised against the N-terminus of SAP102, was used that recognises only a single 102 kDa band on western blots (Fukaya and Watabe, 2000), a generous gift from Masahiko Watanabe (see appendix 1). As figure 4.3c shows, strong staining was observed in wild-type but no signal was detectable from mutant brain sections. These results strongly suggest that no full-length or truncated SAP102 protein is produced in hemizygous mice.

4.4 Discussion

No structural anomalies were observed in PCR or Southern blot analyses of the targeted allele in either ES cells or mice and the mutation was inherited by successive generations in the expected X-linked fashion, suggesting that the SAP102 mice carry the desired homologous recombination event without any other disruption to the genome. Similar analyses of the second, independently targeted line along with a partial phenotypic analysis to ensure agreement with the first line, will need to be performed to further reduce the slight possibility of a linked genomic rearrangement that remains undetected by the methods above.

The brains of male mice hemizygous for the targeted deletion have no detectable SAP102 protein, indicating that the mutation likely produces a null allele.