PolyGene
Transgenetics

Documentation

Project SY10

Fbn2 knock-in mouse model for the human F1670C mutation

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1. Short Introduction

The Fibrillin 2 (Fbn2) is a gene located on mouse chromosome 18, distributed over 65 exons, coding for a protein with 2907 amino acid residues.

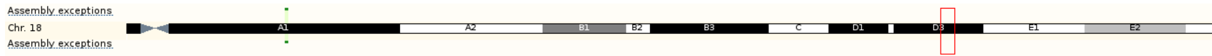


Figure 1: Location of the Fbn2 gene on chromosome 18.

Fbn2 and Fbn1 (Fibrillin 1) are large cysteine-rich glycoproteins found in the extracellular matrix of extensive tissues such as the lung, skin and blood vessels. Fibrillins assemble into microfibrils that then become networks providing structural support for the formation of elastic fibers. These scaffolds of microfibrils also act as targets for growth factors controlling differentiation and morphogenesis (Miller et al., 2010 PlosOne 5(2):e9137). Two mutant mouse lines for Fbn2 have been generated so far, a knockout mouse line and a ENU generated premature stop mutant (Miller et al., 20101).
Within the SYBIL consortium, a knock-in mouse model for the human F1670C mutation is generated (PolyGene project SY10). In mouse, the Phe1663 (F1663) corresponds to Phe1670 in humans, and it is encoded on mouse exon 39.

```
hFBN2        1441 tgdgftcsdvdecaeninlcengqclnvpgayrcecemgftpasdsrscqdidecsfqni
mFbn2        1434 tgdgftcsdvdecaentnlcengqclnvpgayrcecemgftpasdsrscqdidecsfqni

hFBN2        1501 cvfgtcnnlpgmfhcicddgyeldrtggnctdidecadpincvnglcvntpgryecncpp
mFbn2        1494 cvfgtcnnlpgmfhcicddgyeldrtggnctdidecadpincvnglcvntpgryecncpp

hFBN2        1561 dfqlnptgvgcvdnrvgncylkfgprgdgslscnteigvgvsrssccclgkawgnpcet
mFbn2        1554 dfqlnptgvgcvdnrvgncylkfgprgdgslscntevgvgvsrssccclgkawgnpcet

hFBN2        1621 cppvnsteyytlcpggegfrpnpitiiledidecqelpglcqggncintfgsfqcecpqg
mFbn2        1614 cppvnsteyytlcpggegfrpnpitiiledidecqelpglcqggncintfgsfqcecpqg

hFBN2        1681 yylsedtricedidecfahpgvcgpgtcyntlgnytcicppeymqvngghncmdmrksfc
mFbn2        1674 yylseetricedidecfahpgvcgpgtcyntlgnytcicppeymqvngghncmdmrksfc

hFBN2        1741 yrsyngttcenelpfnvtkrmccctynvgkawnkpcepcptpgtadfkticgnipgftfd
mFbn2        1734 yrsyngttcenelpfnvtkrmccctynvgkawnkpcepcptpgtadfkticgnipgftfd

hFBN2        1801 ihtgkavdideckeipgicangvcinqigsfrcecptgfsyndlllvcedidecsngdnl
mFbn2        1794 ihtgkavdideckeipgicangvcinqigsfrcecptgfsyndlllvcedidecsngdnl

hFBN2        1861 cqrnadcinspgsyrcecaagfklspngacvdrnecleipnvcshglcvdlqgsyqcich
mFbn2        1854 cqrnadcinspgsyrcecaagfklspngacvdrnecleipnvcshglcvdlqgsyqcicn
```

F1670
F1663

Figure 2: Comparison of mouse and human Fbn2. The human Phe1670 (F1670) amino acid residue corresponds to mouse F1663 and is located in a highly conserved region of the protein (indicated in red).

The mutation in humans is caused by a T 5009 → G transversion in the codon TTT, coding for phenylalanin, resulting in an exchange for cysteine (TGT). We introduced the same mutation in exon 39 of the mouse (T 4988 → G). In addition, a silent mutation is introduced 2 base pairs upstream of the T 5988 → G mutation that generates an PmlI restriction site which can be used to detect the mutation by combined PCR and restriction analysis. It does not change the amino acid composition of the protein.

```
                         PmlI
5' ccagggtggaaactgtatcaacactttttgggagtttccagtgtgagtgcc 3'
                           g g
3' ggtcccacctttgacatagttgtgaaaaaccctcaaaggtcacactcacgg 5'
                           c c

>   GlnGlyGlyAsnCysIleAsnThrPheGlySerPheGlnCysGluCys   >
                              Cys
```

Figure 3: The mutated Fbn2 allele. The T 4988 → G mutation in exon 39 (yellow) results in an exchange of phenylalanine (Phe) to cysteine (Cys, red).

We applied an FRT-flanked neomycin resistance cassette, which was excised by Flp recombination, upon breeding with germ-line Flp expressing mice (“Flp-deletors”). The neomycin resistance cassette was inserted in an unsuspicious region in intron 39 (compare Fig. 4).

```
mouse  219 agaaacccttgttacattgtccccagca---aattaccgacagcttgcttccattactctccccagaaaacagca---ggcctgcaaaaaaggcaagaaaatcacagt-c
human  207 agaaactctt-tcccattgtcctctaccttgaataatctccaacttaactttattcattttctcagataatggtaaaagttctgtgacaaagccaagaagatatttgagc
rat    204 agaaaaccttgttacattgtccccagca---aattacccacagcttgctttgattactctcctcagaaaacagca---ggcctgcaacaaaggcaagaaaatcacagt-c

mouse  322 agaataaacccagcacctaacggcattcattccttgagttgcacagacgtctgtggtccatacaagatgccatgtttgaaagctgcaatattgactctt-----tggtca
human  316 agaataaacttgccatctaa--gtgtgcgttcatttggatgcatagaaatc--tgattcttacaagatcacactttgaaagctctaatattaactcttatccttagtga
rat    307 ggaataaacccagcaccgaagggcattcattcctttaggtgtacagatgcctgtggtccacacaggatgtcacgtttgaaagctgcgatattgactctt-----tggtca

                                 Exon 39                                                             F1663
mouse  427 t-gattttatcattaatgggttgtctcc--tcttgtagacattgatgaatgtcaggagttaccagggctctgccagggtggaaactgtatcaacactttttgggagtttc
human  422 a-ggtgtttgtttaagtgcactgtctccctttttgtagacattgacgaatgccaggagttaccaggtctctgccagggtggaaactgcatcaacactttttgggagcttc
rat    412 tagattttatcgttgatggactgtctcc--tcttgtagacattgacgaatgtcaggagttaccagggctctgccagggtggaaactgtatcaacactttcgggagcttc

mouse  534 cagtgtgagtgcccacagggttactacctcagtgaggaaacccgcatctgtgaaggtgag--------gaagcctgggatgttccctatgg------------t------
human  531 cagtgtgagtgcccacaaggctactacctcagcgaggatacccgcatctgtgaaggtgagatgaatttgatgcctaaaatgctcagtgctg------------tttaaaa
rat    520 cagtgtgagtgtccacagggttactacctcagtgaagaaacccgcatctgtgaaggtgag--------gaagcctgggctgtcccctaccacagtgtctcccct------

                                   neo          SbfI
                                insertion
mouse  618 -----------------cctg-----------gatcctt-gatcctgcaggagggtctttgagactg-ttaatagcttttttttttcttttttttggagcaatgctgtt
human  629 caaaatggctgctgaggcctgagggaaaggcccatcctccattctacaa-aaggctcaattaagattggttcatagctttttt-----tttttcatatcaatgtagct
rat    616 -----------------cctg-----------g-tcctg-cagccttcaggaggatctgttgagactg-ttaatagcttttctttttt-ttcttctggag----------

mouse  698 catttgtatagtgataaaaccaat-----------ttgcaatgatttctcaaaaaaattttctgagtaaatt--------gattgatgaaccataaat--ggaaa----
human  733 aagttgtatgatgata-gacaaat-----------ta--atttatggctcgttgccataattcactgtattttccaggagggttgattaaatataaattgggaaaatgg
rat    684 catttgtacagtgataaatccaatctgtggctcatttgcaatgattt-acagaaaatttttccaagtaaaat--------gactgat--------------gaaa----

mouse  783 --------gcagaattgcaatatgaaggtggtgaacactga---tatt-----------------tttaagtaggaaca---ggcaaacttcaatgtgactgt-aagtta
human  829 ggtagtgtgaaaacctaaaatattaagtaagttattagtga---aaatattgataagaattacatttacataggaaaaattggtaagctaaaatgtgaccattaactta
rat    767 --------atagaatt-atatatgaag-tagtagacgctgacaataat-----------------tttaagtagcaaca---ggtaaacttaaatgtgaatgt-aagtta

mouse  861 atattggttgatactttctgaatgtcagatataataatttctgaatgatggg---tataatataaagaagttgggatgtcgtacagattc--tttttcc--ccctccccc
human  936 atattg-----------------atcag------taatttttaaatgttggactatatggtatgaagaagttcaaaggttatgcagctgccattcttgcttccatccccc
rat    846 atatttgtcg-----------------------ctaatttctaaatgttgga---tataatataaagaagttggaatgtcgtacagattc--ttt--------ccccacc
```

Figure 4: Comparison of genomic regions flanking Fbn2 exon 39. A comparison of genomic regions from mouse (top), rat (middle) and human (bottom) showed no highly conserved sequences at the potential insertion site for the neomycin cassette in the mouse (SbfI restriction site, indicated in red). The human and rat intron do not contain the SbfII restriction site. Phenylalanine 1663 (F1663) is indicated in black.

2. Retrieval of Genomic Sequences

For this project, we used the BAC RP23-75F07 (compare Fig. 5), covering the genomic region of mouse Fbn2 gene from C57Bl/6 to generate a targeting vector. It encompasses the complete genomic region of Fbn2 with its 65 exons.

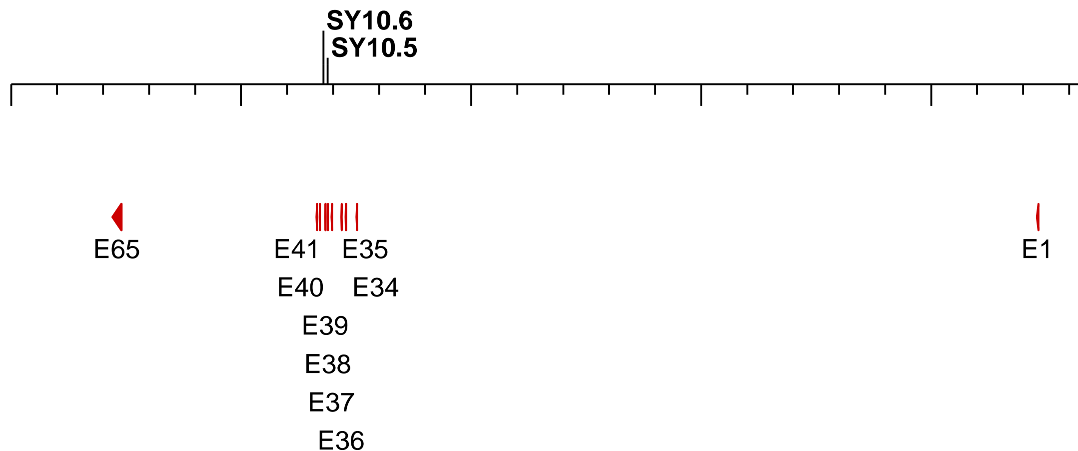


Figure 5: The BAC RP23-75F07. The BAC RP23-75F07 has an insert length of 234 kb and contains exons 1 to 65 of Fbn2 depicted as red arrow heads. The crucial F1663 is located in exon 39. The binding sites of the two primers SY10.5 and SY10.6 surrounding the F1663 are indicated as black bars.

Using the primer pair SY10.5 and SY10.6, we confirmed the identity of the BAC

SY10.5: 5'- AAGGTAAGCCCATCACCAG -3'

SY10.6: 5'- CTGTACGACATCCCAACTTC -3'

amplicon size: 946 bp

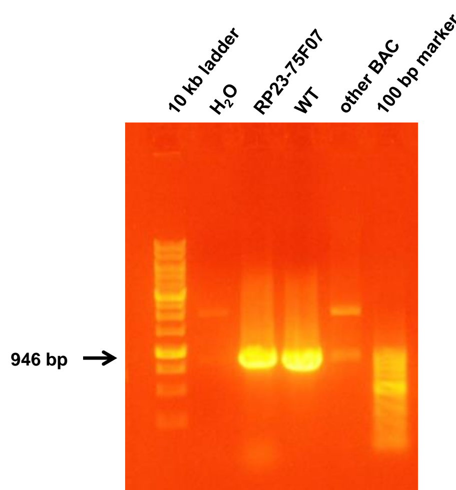


Figure 6: Confirmation of the BAC RP23-75F07. Using primers SY10.5 and SY10.6 a fragment from Fbn2 is amplified by PCR (compare Fig. 5). The BAC RP23-75F07 as well as a genomic probe show the expected signal of 946 bp confirming its identity.

3. Generation of the targeting vector

The BAC RP23-75F07 contains all regions of the Fbn2 gene needed for the generation of the targeting vector. The short and long homology regions as well as an elongated version for the PCR control vector were amplified based on the BAC. For cloning, different restriction sites were added to each PCR fragment. The fragments were cloned into a standard cloning vector and confirmed by complete sequencing.

The mutation in exon 39 of Fbn2 was generated by gene synthesis as a small fragment of 274 bp flanked by the restriction sites AatII and SbfI which are also present in the genome flanking exon 39.

In a first step, the short homology arm was inserted into a vector containing the FRT-flanked neomycin resistance cassette (0008.4a). The restriction sites for NotI and BamHI were used, The product is SY10.2. Analogous, the elongated version of the short homology arm was inserted in the same vector resulting in the PCR control vector SY10.3 CV.

In the third step, the long homology arm was inserted into SY10.2 using AscI and SbfI. The resulting vector is SY10.4.

In the last step, the AatII / SbfI fragment containing the mutation in exon 39 was exchanged in SY10.4 resulting in the final targeting vector SY10.5 TV. All steps are depicted below:

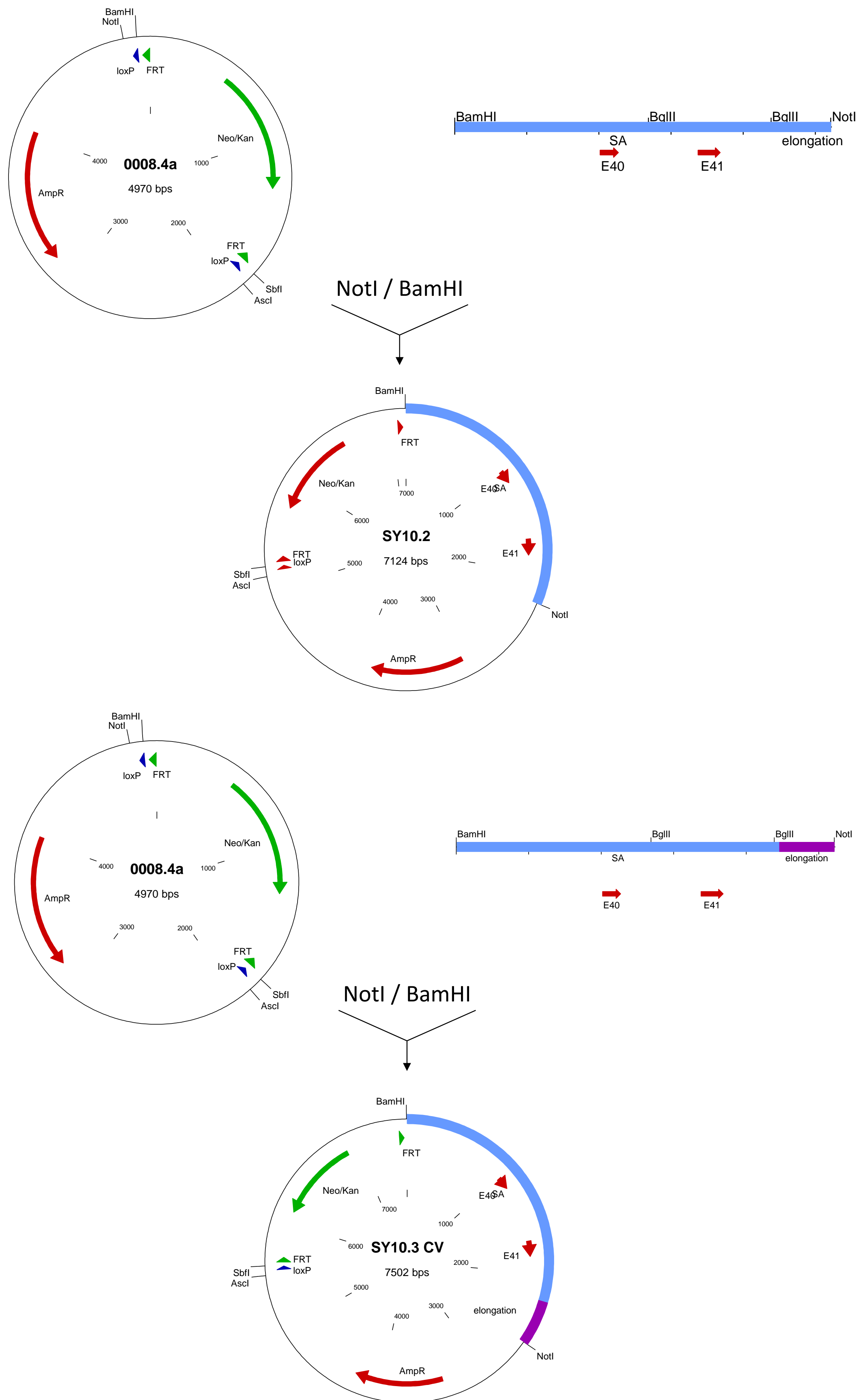


Figure 7: Cloning step 1 and 2. Details see text above.

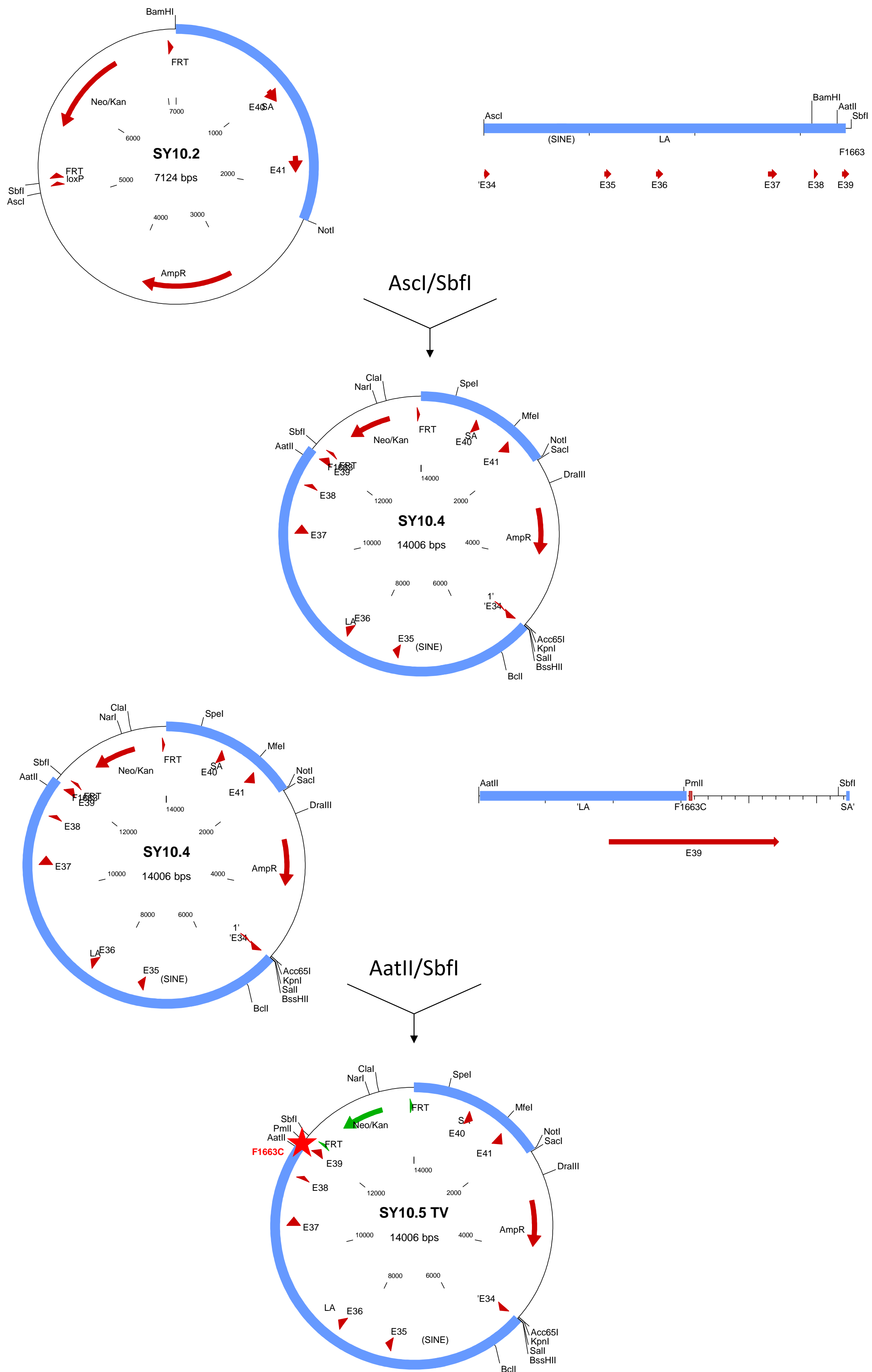


Figure 8: Cloning step 3 and 4. Details see text above.

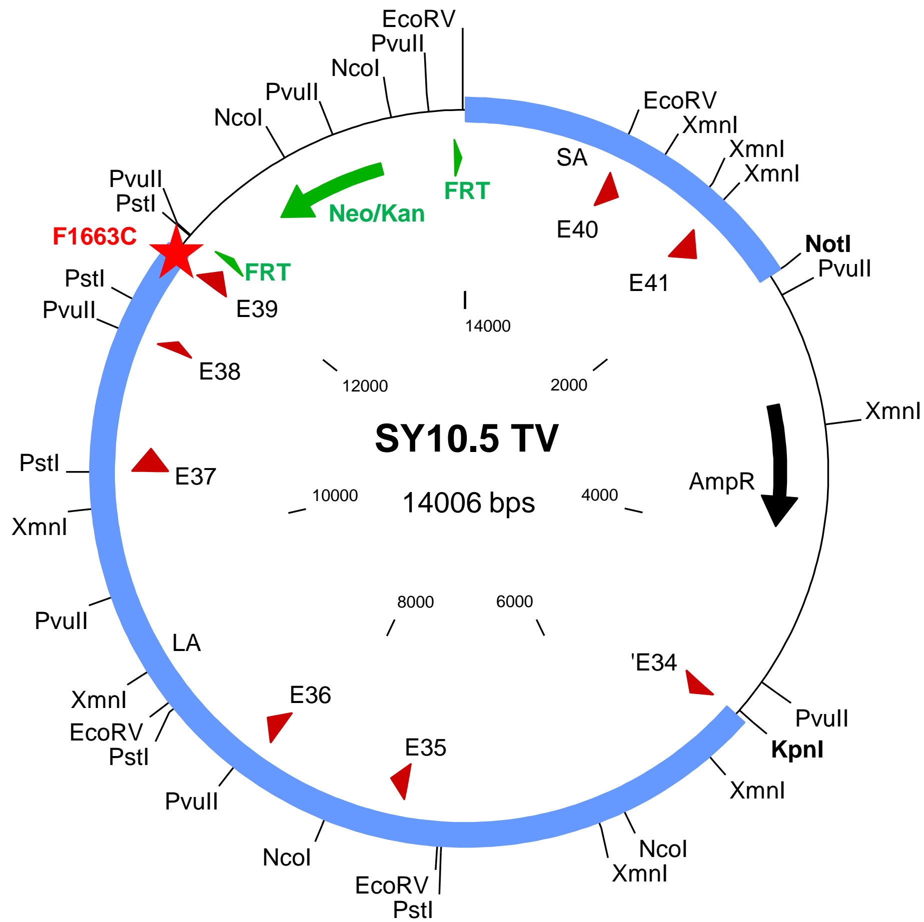


Figure 9: The targeting vector SY10.5 TV. The base exchange (indicated by a red star) in exon 39 of Fbn2 (red arrow heads) is inserted together with the FRT-flanked neomycin resistance cassette (green). The long arm of homology has a length of 6.8 kb. The short arm of homology extends for 2.2 kb. The restriction enzymes KpnI and NotI can be used for linearization prior to electroporation.

The integrity of SY10.5 TV was confirmed by sequencing of all important regions (i.e. the synthesized fragment containing the mutation and all exonic regions) and by restriction analysis. The results are shown below:

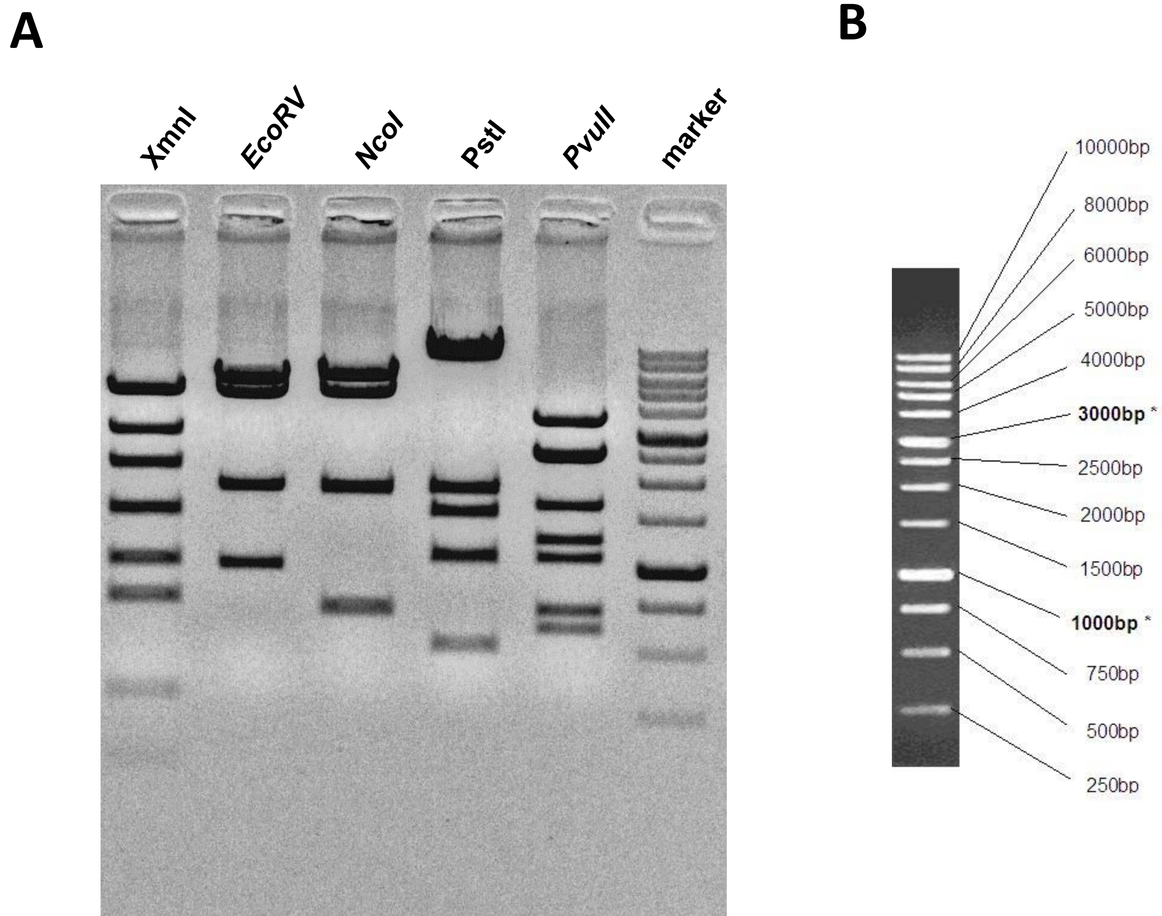


Figure 10: Control digestion of the targeting vector SY10.5 TV. The restriction pattern using XmnI, EcoRV, NcoI, PstI and PvuII confirmed the identity of the targeting vector.

line	enzyme	expected sizes
1.	XmnI	5.0 kb / 3.1 kb / 2.2 kb / 1.5 kb / 1.0 kb / 0.8 kb / (0.3 kb) / (0.1 kb)
2.	EcoRV	6.2 kb / 5.0 kb / 1.9 kb / 1.0 kb
3.	NcoI	6.5 kb / 5.0 kb / 1.8 kb / 0.7 kb
4.	PstI	9.1 kb / 1.8 kb / 1.5 kb / 1.1 kb / 0.5 kb
5.	PvuII	3.6 kb / 2.6 kb / 2.5 kb / 1.6 kb / 1.3 kb / 1.1 kb / 0.7 kb / 0.6 kb
6.	10 kb Marker	10 kb / 8 kb / 6 kb / 5 kb / 4 kb / 3 kb / 2.0 kb / 1.5 kb / 1 kb / 0.8 kb / 0.6 kb / 0.5 kb

The restriction pattern shows restriction fragments of precisely the expected sizes and confirms the integrity of the targeting vector SY10.5 TV as it is drawn in Fig. 9.

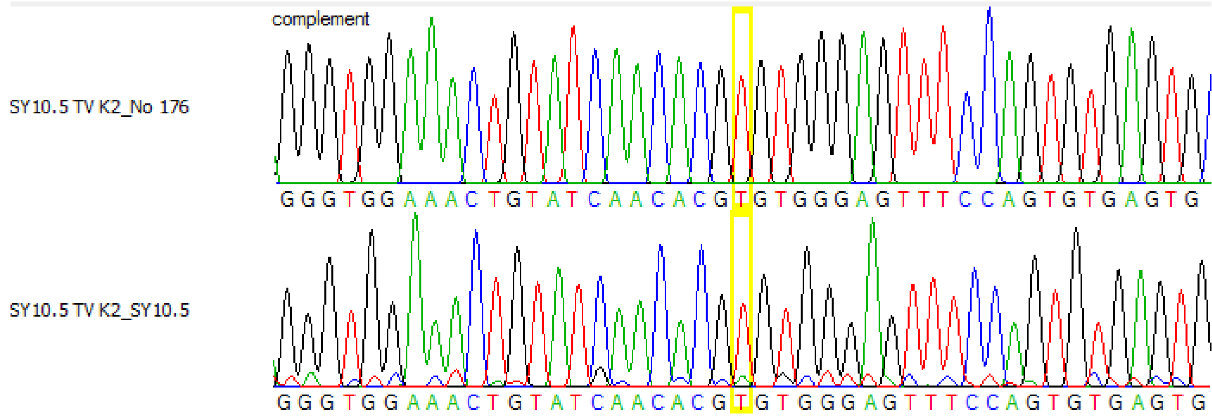


Figure 11: Sequencing result for the site flanking the F1663C mutation. The Chromatogram of two different sequencing reactions is shown covering the region around the F1663C mutation. In the upper panel, the corresponding DNA sequence and the resulting amino acids are in the targeting vector SY10.5 TV indicated.

Restriction and sequencing analyses confirmed the correct targeting vector SY10.5 TV.

4. Transfection of embryonic stem cells

To modify the Fbn2 locus, 20 ug of the targeting vector SY10.5 TV were linearized using NotI, and electroporated into 1.8 x 10^7 C57Bl/6-derived ES cells. G418 (0.3 mg/ml) was used to select for stable transfection. After 8 days of selection, a total of 4 times 96 clones was picked and analysed via PCR analysis.

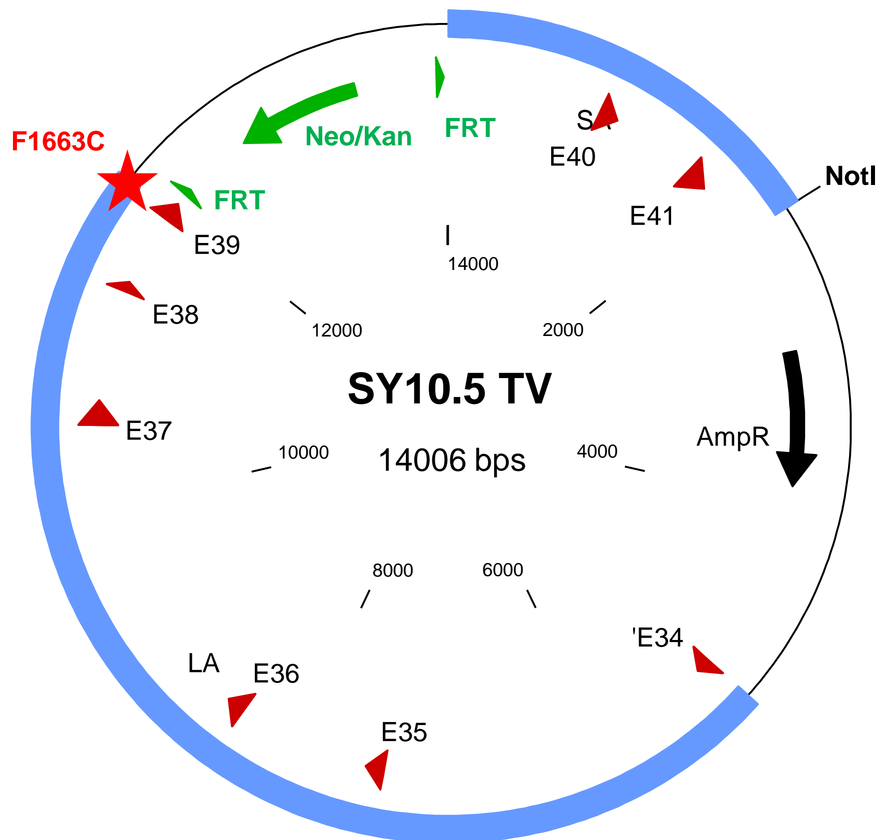


Figure 12: The targeting vector SY10.5 TV. The restriction site NotI used for linearization is indicated.

5. Analysis of ES cell clones

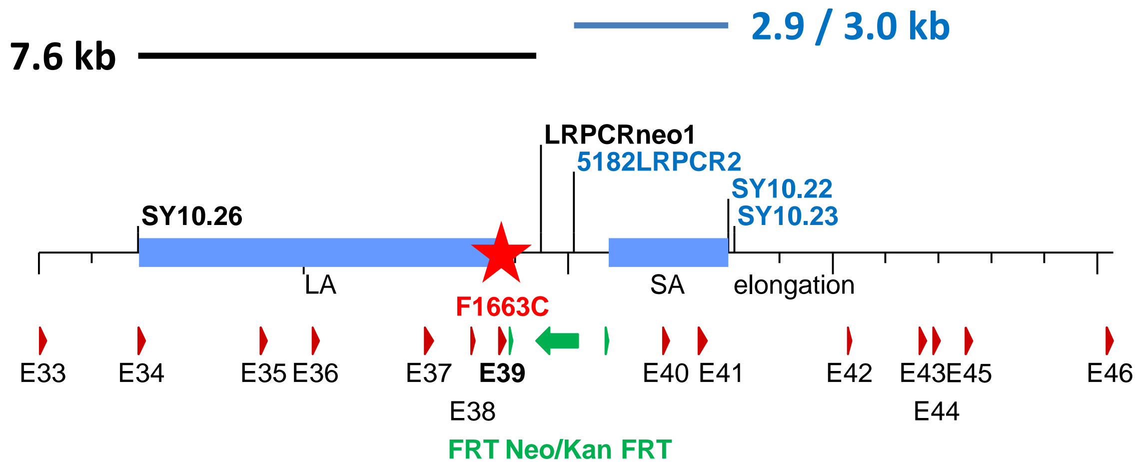


Figure 13: Schematic drawing of the targeted Fbn2 allele. The homologous regions (*SA:* short arm of homology; *LA:* long arm of homology) used for recombination are depicted as blue boxes. The F1663C mutation (red star) is inserted in exon 39 (red arrow head) in the targeted allele (TG) via homologous recombination together with an FRT-flanked neomycin cassette (green). The primers used for screening and confirmation PCR and the corresponding fragments are indicated in black and blue.

5.1 Screening for positive ES cell clones

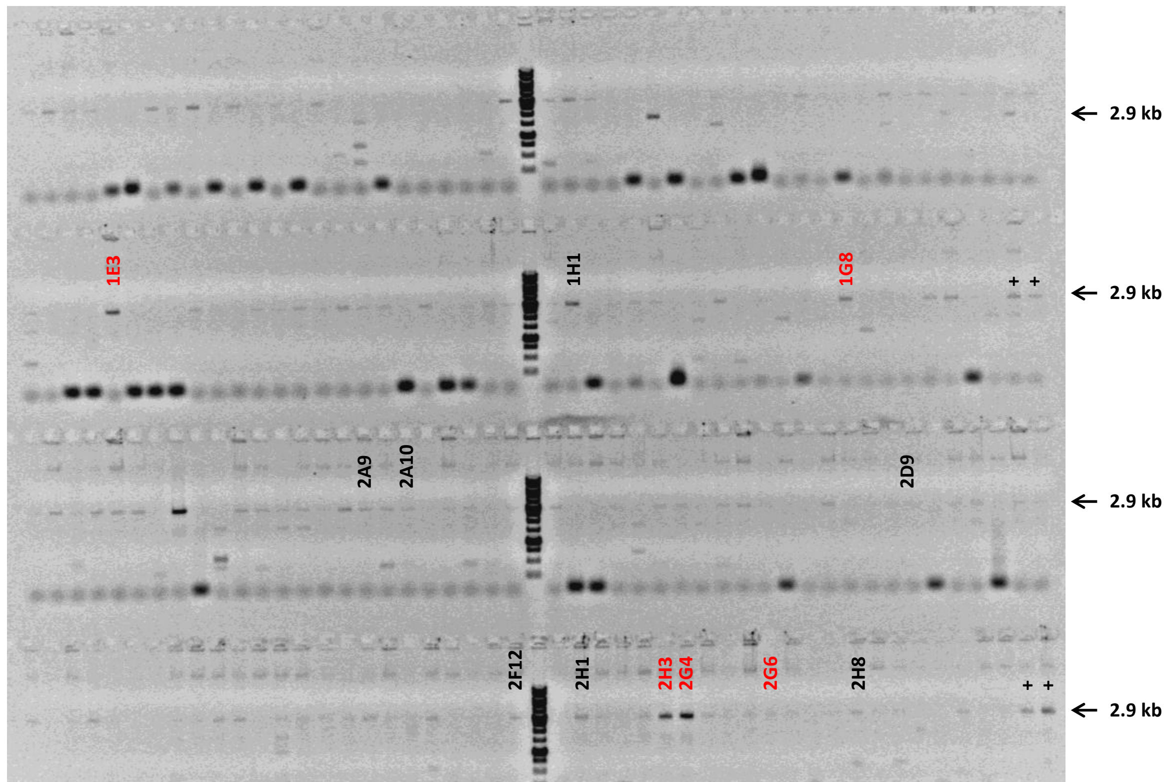
4 x 96 clones were isolated after selection with G418, and lysates of 2 x 96 clones were tested via two different screening PCR analysis. The following primers were used:

5182LRPCR2: 5'- GTTGTGCCCAGTCATAGCCGAATAG - 3'
SY10.22: 5'- CTGCTTGGTTCCCAAAGTG - 3'

or

5182LRPCR2: 5'- GTTGTGCCCAGTCATAGCCGAATAG - 3'
SY10.23 5'- GGAATGGCCAGTCTTGTTTG - 3'

Expected amplicons: 2.9 or 3.0 kb



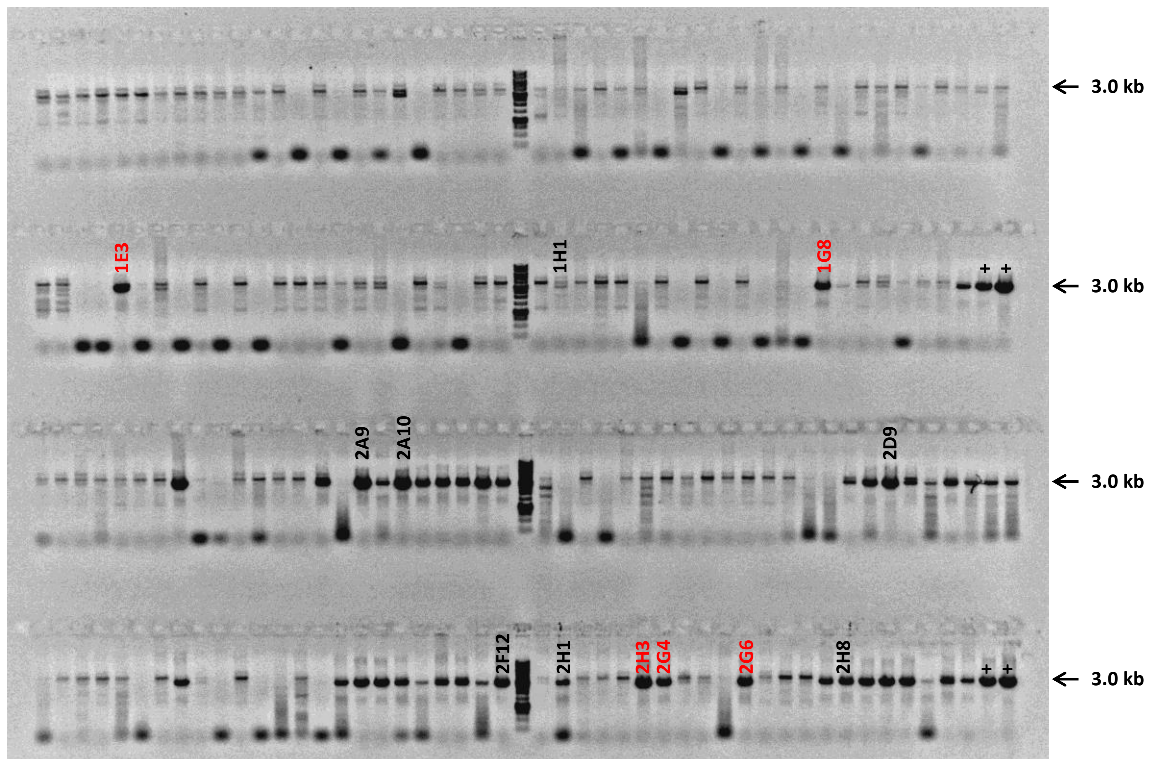


Figure 14: Screening PCR analyses. Lysates from 2 x 96 ES cell clones were tested for correct gene targeting using two different primer combinations. Upper panel: The primer combination 5182LRPCR2 / SY10.22 revealed several potential positive clones indicated by a signal at 2.9 kb. Lower panel: The primer combination 5182LRPCR2 / SY10.23 revealed a number of potential positive ES cell clones by a signal at 3.0 kb. 1 pg and 10 pg of the PCR test vector were used as positive controls (+). Please note, that positive clones for one PCR are not in all cases positive for the second PCR. Clones confirmed in all analyses are indicated in red.

Result: Screening of the first 2 x 96 ES cell clones revealed many potential positive clones by a signal of either 2.9 or 3.0 kb. 12 clones were selected that showed signals in both PCR analyses. The clones were expanded, frozen and DNA was prepared. This DNA was used for further testing. As a backup plates 3 and 4 were frozen completely.

5.2 Confirmation of potential positive clones

DNA from the 12 selected clones was used for the same PCR analysis using primers 8152LRPCR2 and SY10.22. The result is shown below:

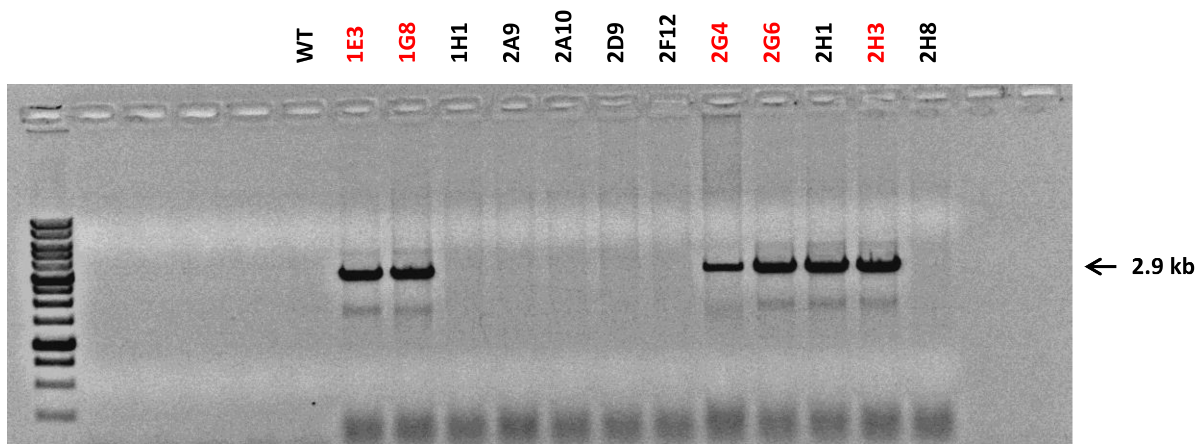


Figure 15: Confirmation PCR analyses. DNA from 12 potential positive ES cell clones was tested for correct gene targeting using the primer combination 5182LRPCR2 / SY10.22. A signal at 2.9 kb indicates correct homologous recombination

Result: PCR analysis using DNA from 12 potential positive clones confirmed 6 clones by a signal of 2.9 kb.

5.3 Confirmation on the long homology arm

For confirmation of correct homologous recombination with the Fbn2 locus, long-range PCR was performed using the following primers:

SY10.26 5'- ATTCGGCAGATGTTGACGAGTG -3'

LRPCRneo1 5'- AATGGGCTGACCGCTTCCTCGTGCTTT -3'

Expected signal: 7.6 kb

The result is shown below:

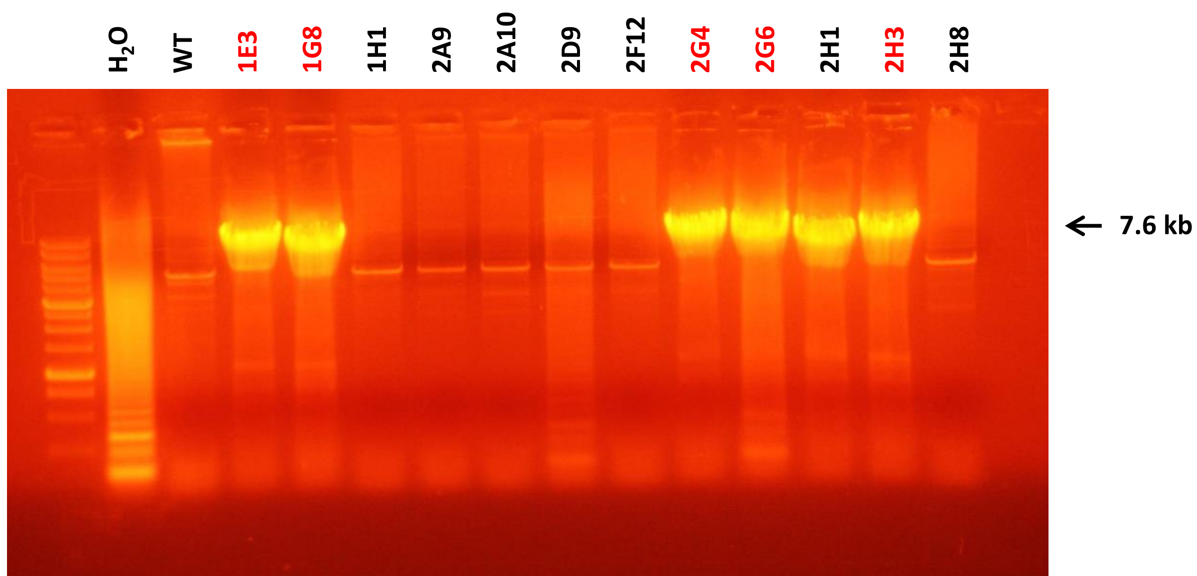


Figure 16: Long-range PCR analyses. DNA from 12 potential positive ES cell clones was tested for correct gene targeting using the primer combination LRPCRneo1 / SY10.26. A signal at 7.6 kb indicates correct homologous recombination.

Result: PCR analysis using DNA from 12 potential positive clones confirmed 6 clones by a signal of 7.6 kb. To confirm the insertion of the F1663C mutation in exon 39, the PCR product was eluted form the gel and digested with the restriction enzyme PmlI. By insertion of the F1663C mutation this site is generated in exon 39. The result is shown below:

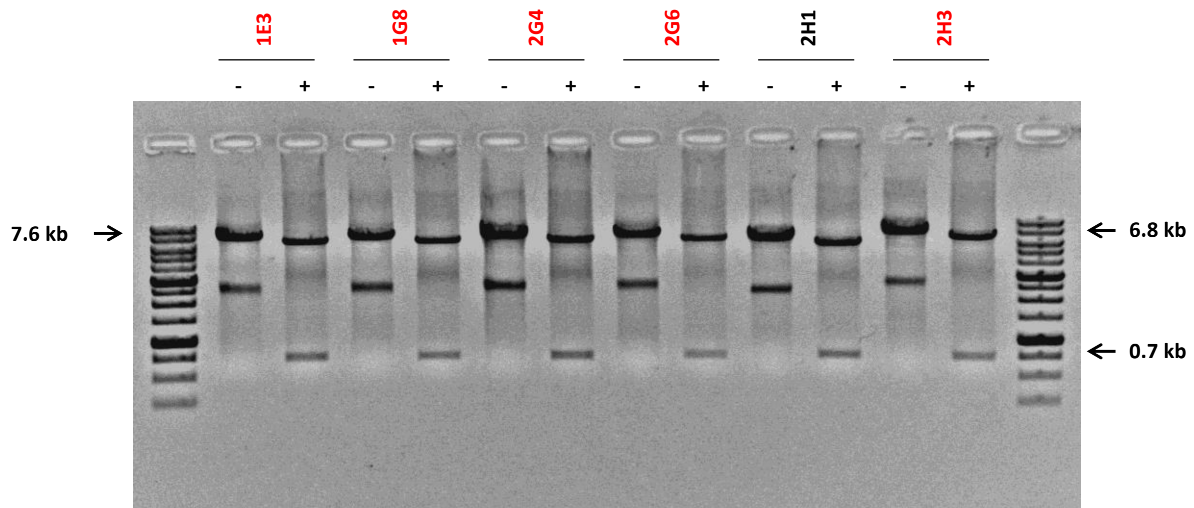


Figure 17: Restriction analysis of the long-range PCR products. The PCR product from 6 PCR positive ES cell clones (7.6 kb, -) was digested by the restriction enzyme PmlI (+). If the F1663C mutation is present in the PCR product, it results in two fragments of 6.8 and 0.7 kb. If the mutation is not present the PCR product is uncut. Please note that the purified PCR product (-) contains an additional unspecific signal at about 2.5 kb. This could be caused by a secondary structure only present in this buffer. After digestion with PmlI and in the corresponding buffer this signal disappeared. Clones indicated in red were confirmed positive in all analyses.

Result: Digestion of the long-range PCR products with the restriction enzyme PmlI revealed that all clones tested do contain the F1663C mutation, indicated by the 0.7 kb fragment.

5.4 Southern blot analyses

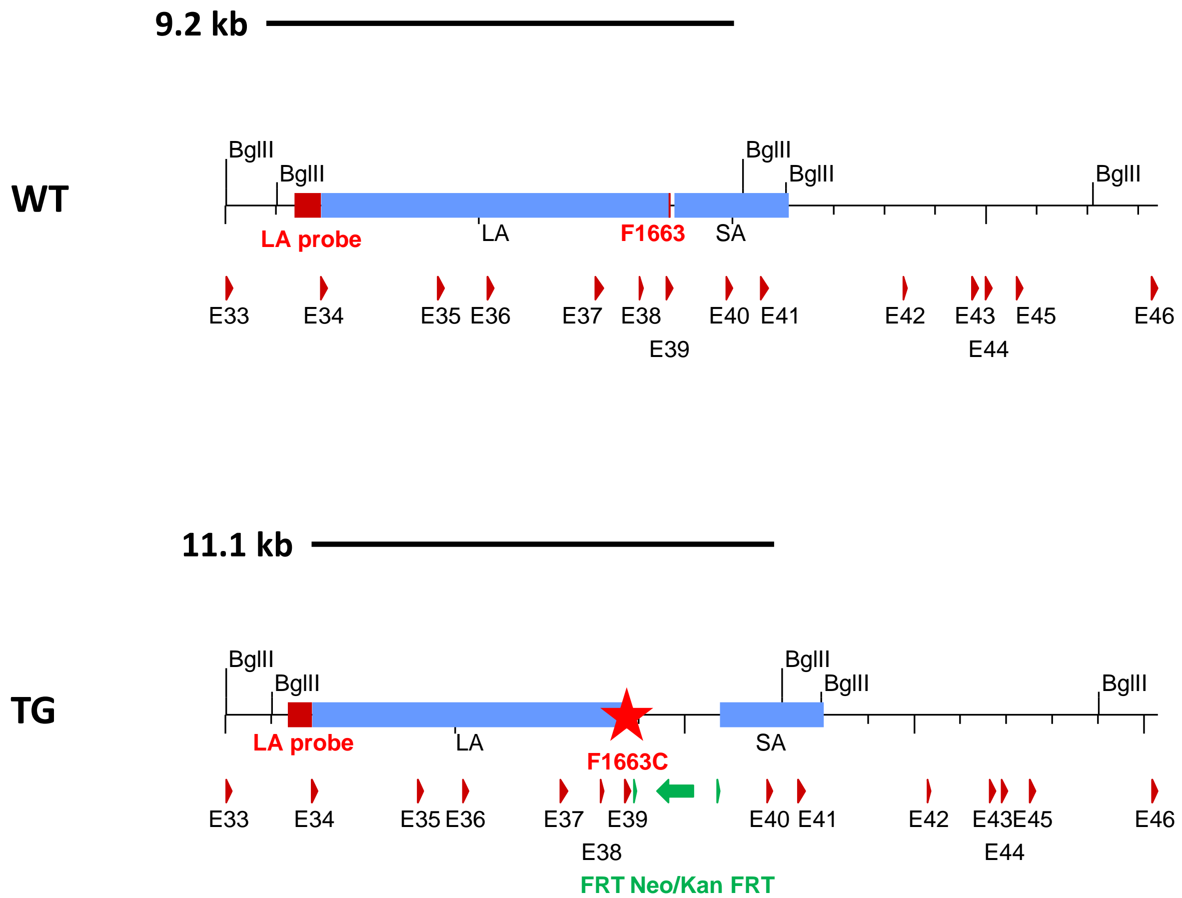


Figure 18: Schematic drawing of the different Fbn2 alleles. The homologous regions (*SA:* short arm of homology; *LA:* long arm of homology) used for recombination are depicted as blue boxes. In comparsion to the wild typ allele (WT), the F1663C mutation (red star) is inserted in exon 39 (red arrow head) in the targeted allele (TG) via homologous recombination together with an FRT-flanked neomycin cassette (green). The restriction enzyme BglII and a 5' external probe (LA probe) were used for Southern blot analyses. The expected sizes for are indicated as black bars.

For confirmation of correct homologous recombination with the Fbn2 locus, Southern blot analysis was performed using BglII digested DNA. DNA was hybridized with a 5' external probe. The probe has a size of 494 bp and was generated using the following primers:

SY10.24 5'- CGGCTCAGTAAACACTGGTC-3'

SY10.25 5'- GACGTCTCCGTTAAGTTC-3'

Hybridization results in a 9.2 kb signal for the wild type allele and a 11.1 kb signal for the correctly targeted allele. The result of the analysis is shown below:

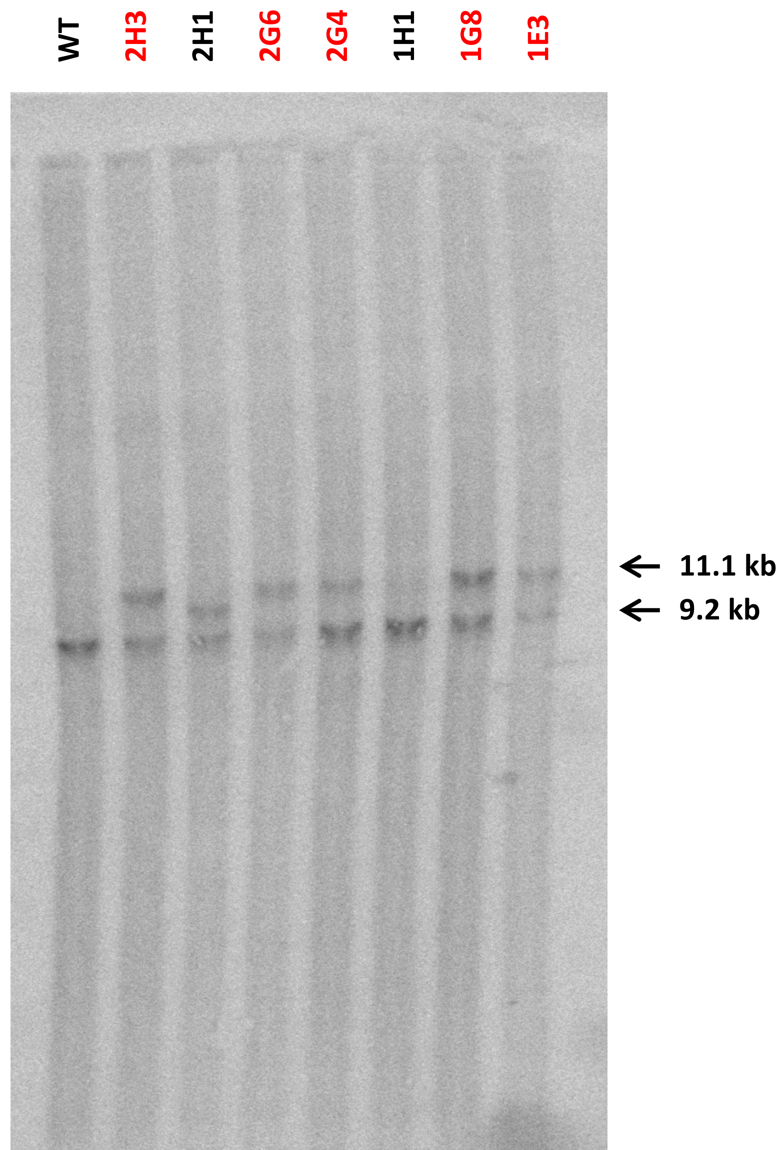


Figure 19: Confirmation Southern blot analyses. DNA from 7 potential positive ES cell clones was digested using the restriction enzyme BglII. DNA from another project served as wild type control (WT). A 5' external probe was used for detection of the Fbn2 allele. The analysis using the 5' external probe confirmed correct homologous recombination within 5 clones (indicated in red). This is indicated by a signal of 11.1 kb representing the correctly targeted allele. The wild type signal has a size of 9.2 kb. One clone shows only the wild type signal (1H1) and one clone (2H1) shows a second signal that is smaller than the expected 11.1 kb.

Result: Southern blot analyses confirmed correct homologous recombination for 5 clones tested (compare Fig. 19).

5.5 Discussion and Summary

Taken together, we could identify a large number of potential positive ES cell clones via two different screening PCR analyses. 12 clones positive for both PCRs were chosen and expanded. Out of these 12 clones 6 could be confirmed by the same screening PCR and by long-range PCR. This indicates that the two screening PCRs resulted in a large number of false positive results when done on cell lysates. The same PCR on DNA gave clear and reliable results that were confirmed by other analyses.

Long-range PCR combined with restriction analysis confirmed 6 clones for correct homologous recombination on the long homology arm and for the presence of the F1663C mutation. Out of these 6 clones, 5 were confirmed by Southern blot analysis.

These five clones were used for injection into blastocysts.

6. Blastocyst injection and breeding

On May 12, 2016, clones 2H3, 1E6, 1G8, and 2G4 (C57Bl/6N derived) were injected into 80 blastocysts from grey C57Bl/6 mice. 73 surviving blastocysts were transferred into four CD-1 foster mice. From this injection, five chimeric mice (~50-100%) were born on May 31, 2016. Three chimeras (50-80%) were originating from clone 1G8, whereas two highly chimeric mice (100%) were derived from clone 2G4. Additionally, 4 grey mice were born. The chimeric mice are depicted in Fig. 1 and were mated to grey Flp-deleter mice (C57Bl/6N derived).

Figure 20: Chimeras 24 - 28 from clones 1G8 and 2G4.

7. Screening of offspring, following the mating of chimeras to Flp-deleter mice

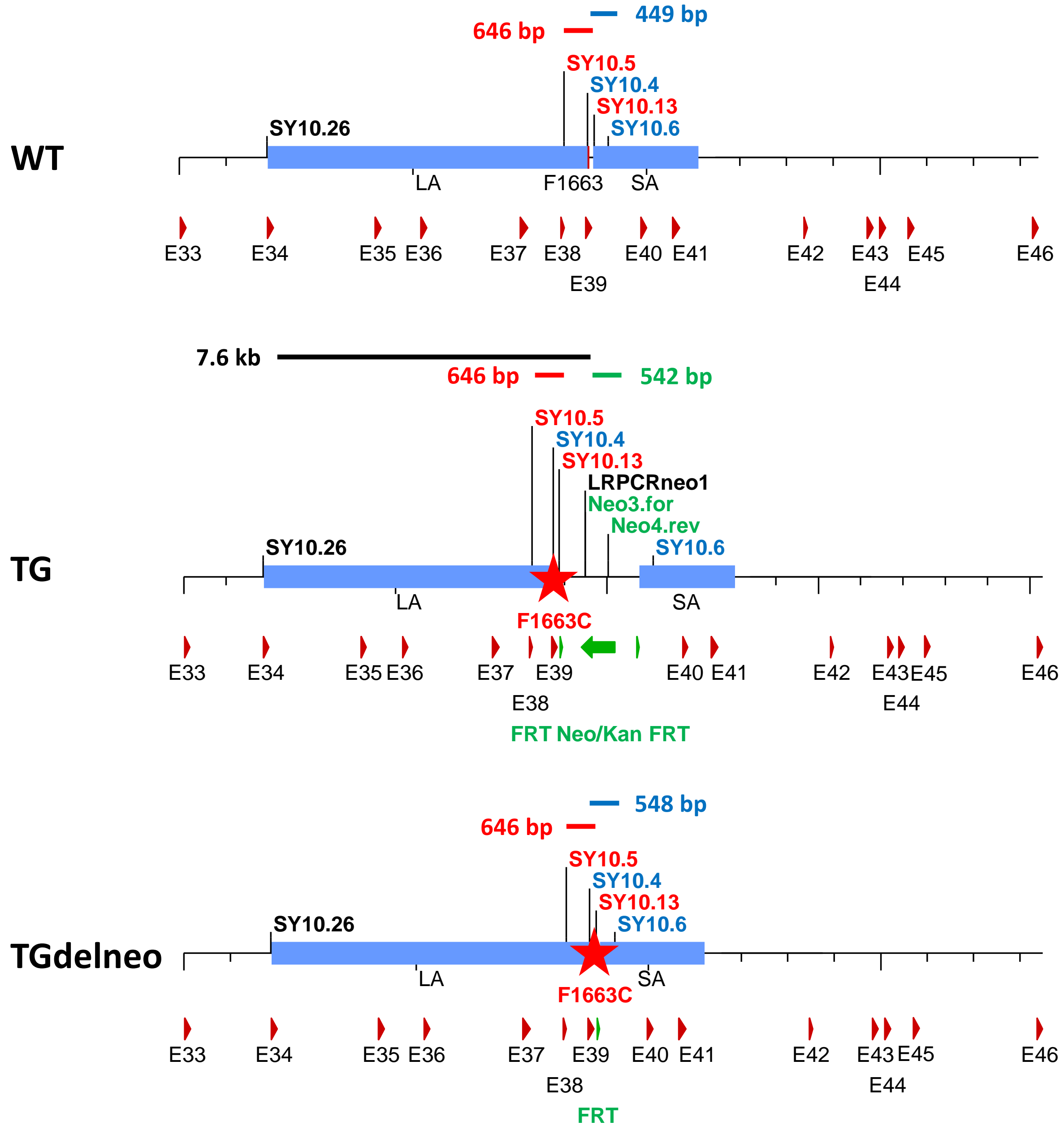


Figure 21: The different Fbn2 alleles. Comparison of the Fbn2 wild type (WT, upper panel), the targeted allele (TG, middle panel) and the targeted allele after deletion of the neomycin cassette (TGdelneo, lower panel). The binding sites of the primers used for genotyping are indicated in relation to the Fbn2 exons (red arrows) and the FRT flanked neomycin cassette (green). The corresponding PCR fragments are indicated in red (for mutation), green (for neo presence), and blue (for neo deletion) and black (for detection of homologous recombination). The F1663C mutation inserted in exon 39 of Fbn2 is indicated as a red star.

7.1 Screening for the deletion of the neomycin resistance cassette

Black offspring from the mating of the chimeras with grey Flp-deleter mice was screened for the Flp-mediated deletion of the neomycin cassette which is shown by the presence of the remaining FRT site (compare Fig. 21).

The primer combination SY10.4 and SY10.6 can be used for screening. Positive animals are germ line transmitters of the targeted Fbn2 allele after deletion of the neomycin cassette.

SY10.4: 5'- AAAACGGGTGGAAACTGTATCAACAC -3'

SY10.6: 5'- CTGTACGACATCCCAACTTC -3'

Expected amplicons:

Wild type: 449 bp

Targeted: 548 bp

The result of a representative PCR screening of the F1 generation is shown below:

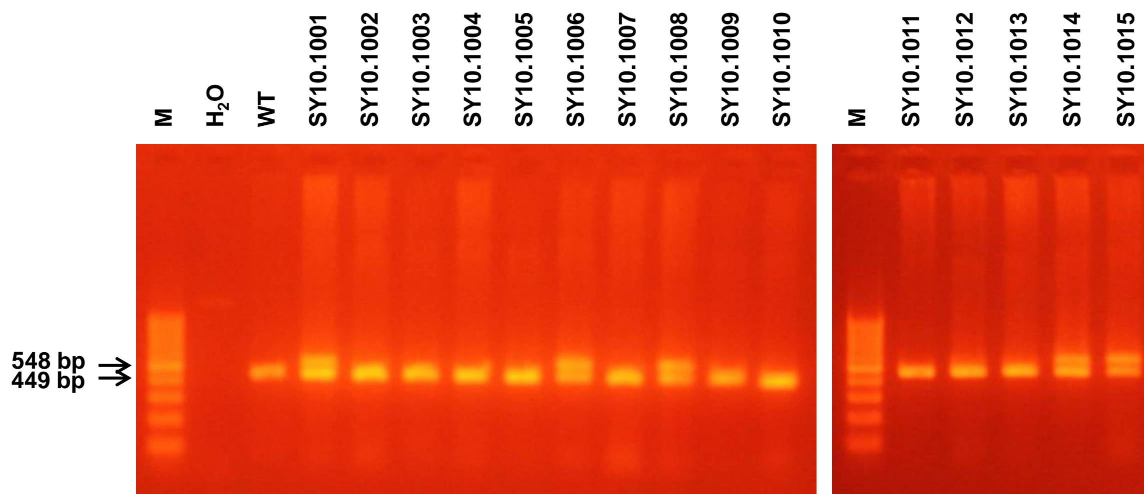


Figure 22: Genotyping PCR for the targeted allele after deletion of the neomycin cassette. The representative result of screening F1 generation mice for the presence of the remaining FRT site left after Flp-mediated deletion of the neomycin cassette in the Fbn2 locus using primers SY10.4 and SY10.6 is shown.

Animals 1001, 1006, 1008, 1014, 1015 and some more pups from the F1 generation (not shown) are positive for the targeted allele after deletion of the neomycin cassette, indicated by the presence of the 548 bp PCR product.

7.2 Screening for the presence of the neomycin resistance cassette

Additional mice from the F1 generation were screened for the presence of the neomycin cassette in the targeted allele, to identify germ-line transmitted offspring that still contain the neomycin cassette.
The primer combination Neo3-for / Neo4-rev can be used for screening. Positive animals are germ line transmitters of the targeted Fbn2 allele before deletion of the neomycin resistance cassette.

Neo3-for: 5'- CAAGCTCTTCAGCAATATCACGGG -3'
Neo4-rev: 5'- CCTGTCCGGTGCCCTGAATGAACT -3'

Expected amplicon: 542 bp

The result of a representative PCR screening of the F1 generation is shown below:

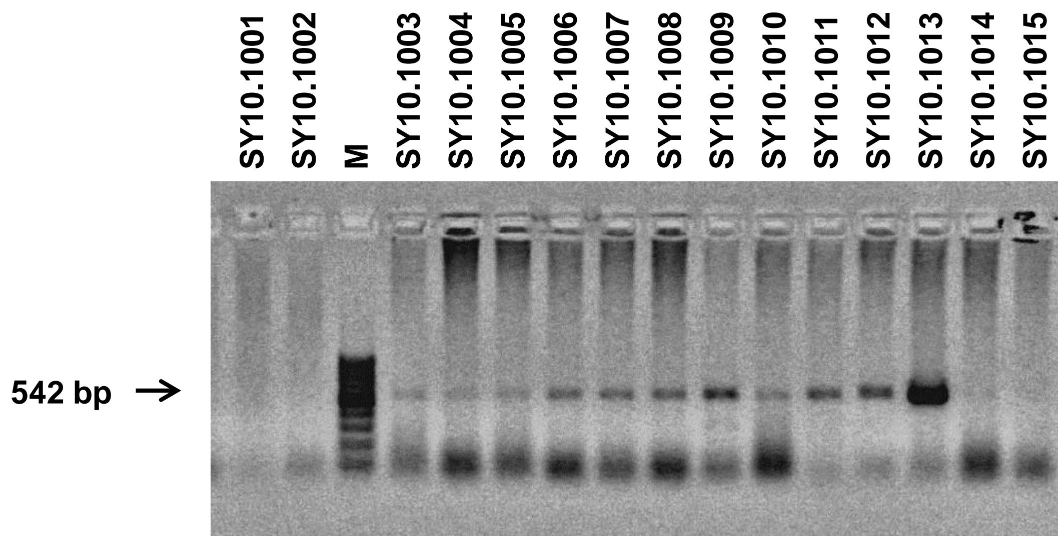


Figure 23: Genotyping PCR for the targeted allele before deletion of the neomycin resistance gene. The representative result of screening F1 generation mice for the presence of the neomycin resistance cassette in the Fbn2 locus using primers Neo3-for and Neo4-rev is shown. Please note a much weaker background signal present in almost all samples. The positive mouse shows a much stronger signal.

Animal 1013 and some more mice from the F1 generation (not shown) were positive for the targeted allele before deletion of the neomycin resistance gene, indicated by the presence of the 542 bp PCR product.

7.3 Detection of the mutation in mice

The mutation in exon 39 of Fbn2 that was introduced into the locus on chromosome 18 is located about 100 bp upstream of the insertion site of the selection cassette. To test for the presence of the mutation, one can make use of the primer combination SY10.5 and SY10.13 for amplification of the corresponding genomic fragment.

SY10.5: 5'- AAGGTAAGCCCATCACCAG -3'

SY10.13: 5'- CCCTCCTGCAGGATGAAGGATG -3'

Expected amplicon: 646 bp

This fragment was sequenced:

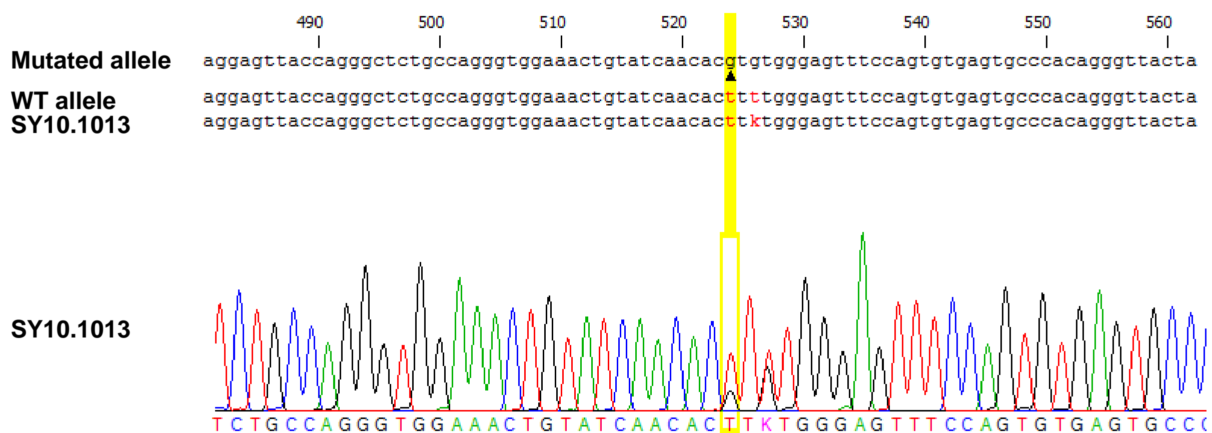


Figure 24: Analysis for the presence of the F1663C mutation in the Fbn2 locus: DNA from ear biopsies of the one putative positive mouse from F1 generation (SY10.1013) was used to test for the presence of the mutation within the Fbn2 locus. Primers SY10.5 and SY10.13 were used to generate PCR fragments that were subsequently sequenced. The two upper rows contain the mutated and the wild type sequence. The third row contains the sequencing result and the corresponding chromatogram below.

Result: As can be seen in figure 24, the combined PCR and sequencing analysis confirmed that the mouse analysed contains the F1663C mutation in exon 39 of Fbn2.

7.4 Confirmation of homologous recombination

Additionally, DNA was prepared from one neo positive mouse (SY10.1013) and was tested for the correct insertion of the targeted allele in the genome. The primer combination LRPCRneo1 and SY10.26 can be used for screening. LRPCRneo1 binds to a region in the neomycin cassette, whereas SY10.26 is specific for a region in exon 34 of Fbn2 upstream of the long homology arm (compare chapter 5.3 ES cells).

Expected amplicon: 7.6 kb

The result of a representative PCR screening of the F1 generation is shown below:

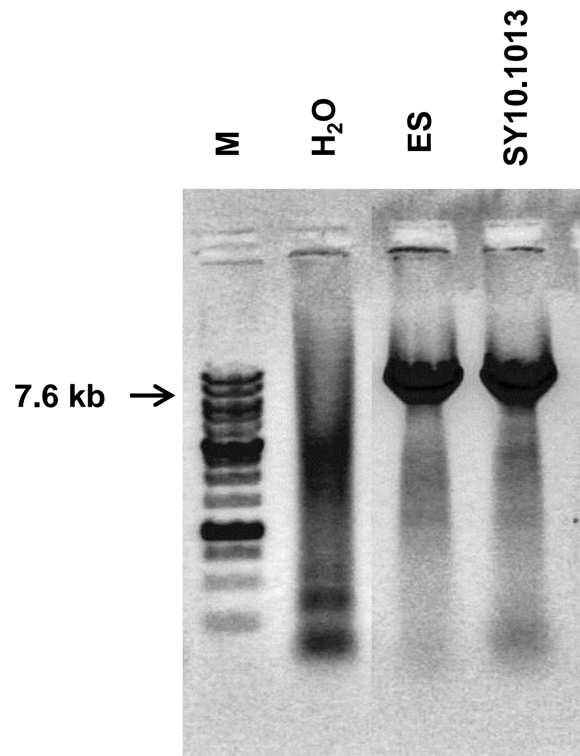


Figure 27: Genotyping PCR for the targeted allele in the mouse genome. The result of confirming SY10.1013 for the presence of the targeted allele in the mouse genome using primers LRPCRneo1 and SY10.26 is shown. Water (H_2O) and DNA from a positive ES cell clone (ES) served as controls.

Animal 1013 from the F1 generation was confirmed positive for correct homologous recombination in the Fbn2 locus by the presence of the 7.6 kb PCR product.

7.4 Screening for the presence of the Flp-recombinase allele

Additionally, the mice from mating of chimeric mice with grey Flp-deleter mice were screened for the presence of the Flp-recombinase. The primer combination SD24 / SD25 can be used for screening.

SD24: 5'- CTAATGTTGTGGGAAATTGGAGC -3'

SD25: 5'- CTCGAGGATAACTTGTTTATTGC -3'

Expected amplicon:

Flp-allele: 568 bp

The result of a representative PCR screening of the F1 generation is shown below:

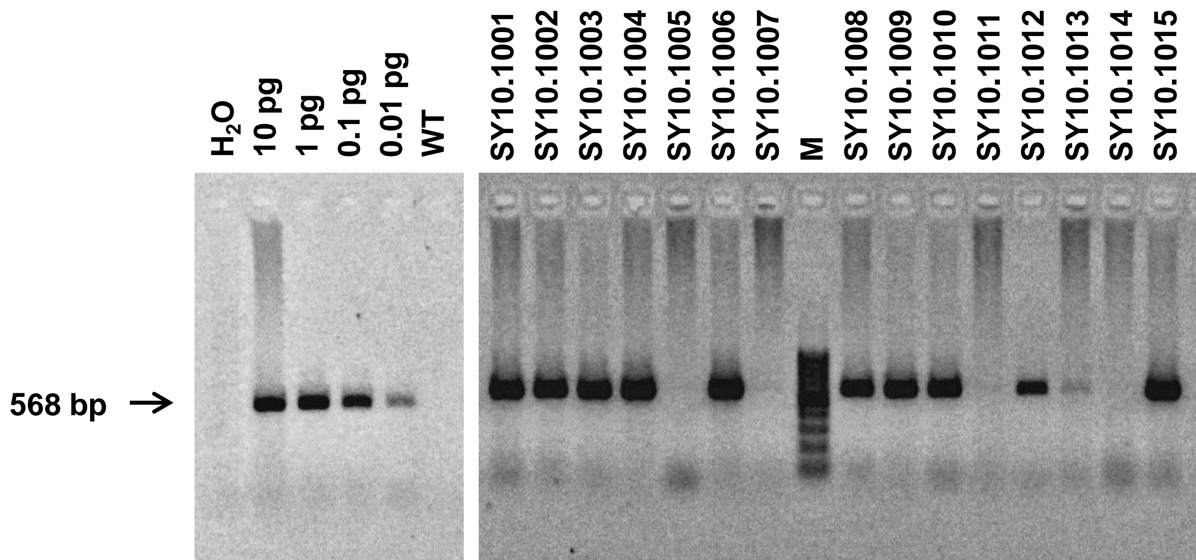


Figure 25: Genotyping PCR for the Flp-recombinase gene. The representative result of screening generation mice for the presence of the Flp-recombinase gene using primers SD24 and SD25 is shown.

Animals 1001, 1002, 1003, 1004, 1006, 1008, 1009, 1010, 1012, 1015 and several more mice from the F1 generation are positive for the Flp-recombinase transgene indicated by the presence of the 568 bp PCR product.

7.5 Summary

Breeding of the chimeras to grey Flp-deleter mice resulted in several positive mice containing the targeted allele. Most already lost the neomycin cassette due to activity of the Flp-recombinase. A list of all genotyping results is shown in Table 1 below.

animal	sex	neo	delneo	screening	mutation	Flp
1001	female	-	positive	n.t.	n.t.	positive
1002	male	-	-	n.t.	n.t.	positive
1003	female	-	-	n.t.	n.t.	positive
1004	female	-	-	n.t.	n.t.	positive
1005	female	-	-	n.t.	n.t.	-
1006	female	-	positive	n.t.	n.t.	positive
1007	female	-	-	n.t.	n.t.	-
1008	female	-	positive	n.t.	n.t.	positive
1009	female	-	-	n.t.	n.t.	positive
1010	female	-	-	n.t.	n.t.	positive
1011	male	-	-	n.t.	n.t.	-
1012	male	-	-	n.t.	n.t.	positive
1013	male	positive	-	positive	positive	-
1014	male	-	positive	n.t.	n.t.	-
1015	male	-	positive	n.t.	n.t.	positive

Table 1: Summary of genotyping results for the F1 generation. The results of different genotyping PCRs for 15 mice from the F1 generation is shown. Mice indicated in red are suited for shipment to Antwerp.