Supplemental data

Biallelic mutations in the ubiquitin ligase *RFWD3* cause Fanconi anemia

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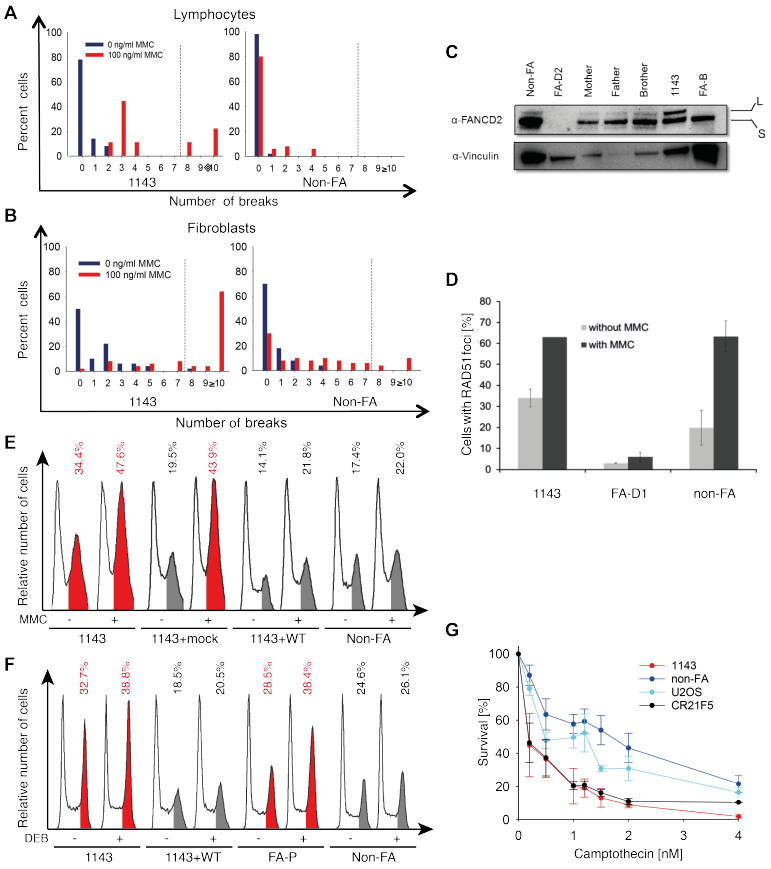
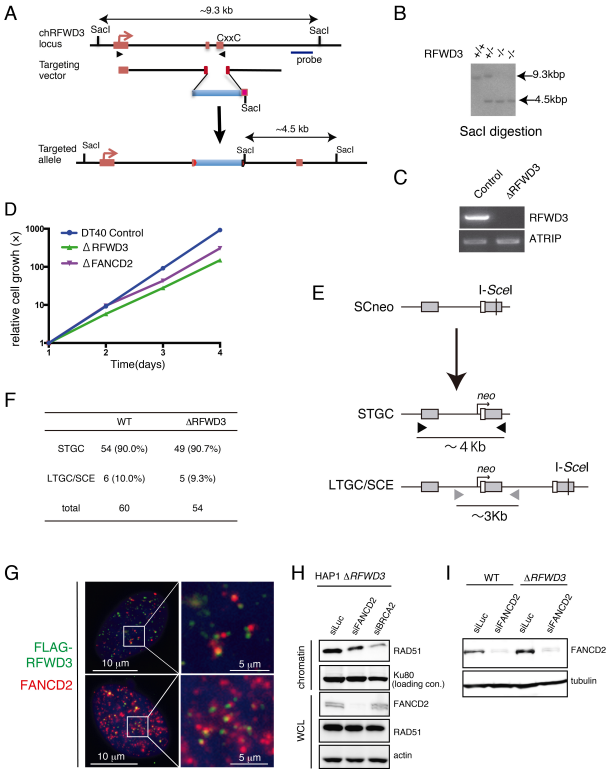


Supplemental Figure 1. Extended FA phenotype of 1134 cells (related to Figures 1 and 2).
(A) Histograms reflecting proportions of cells with the indicated number of chromosomal breaks per metaphase from lymphocyte cultures, blue without, red after exposure to MMC. Dashed lines delimit high rates (>7). 50 metaphases each were scored.
(B) Histograms reflecting proportions of cells with the indicated number of chromosomal breaks per metaphase from fibroblast cultures, blue without, red after exposure to MMC. Dashed lines delimit high rates (>7). 50 metaphases each were scored. The 1143 plot is reused in Figure 1C.
(C) Immunoblot analysis of FANCD2 monoubiquitination of *RFWD3*-mutated (1143), 1143's family members, *FANCD2*-mutated, *FANCB*-mutated and normal control lymphoblasts. Cells were exposed to 40 ng/ml MMC for 16 h.
(D) Proportion of RAD51 foci-positive nuclei in 1143, FA-D1 and non-FA fibroblasts, detected by anti-RAD51 staining without (gray) and with (black) prior exposure of cells to MMC (40 ng/ml, 16 h). Data represent mean ± SD. *N*=3.
(E) Cell-cycle analysis without or with exposure to MMC of 1143, 1143+mock, 1143+WT-RFWD3 and non-FA fibroblasts. Increased G2 compartment size of 1143 and 1143+mock cells (normal <28%) is highlighted in red, normal size is shown in gray.
(F) Cell-cycle analysis without or with exposure to MMC of 1143, 1143+WT-RFWD3, FA-P and non-FA fibroblasts. Increased G2 compartment size of 1143 and FA-P cells (normal <28%) is highlighted in red, normal size is shown in gray.
(G) Dose-response curves of CRISPR clone CR21F5 with targeted RFWD3 compared to parental U2OS, 1143 and non-FA cells, exposed to camptothecin at the indicated concentrations. Data represent mean ± SEM; *N*=5. The 1143 and non-FA curves are reused in Figure 1F.



Supplemental Figure 2. Generation and characterization of RFWD3- deficient cellular models (related to Figures 3 and 4).

(A) Partial map of the chicken *RFWD3* locus, the gene disruption vector and the configuration of the targeted allele. An inserted neo-cassette is shown in light blue, a probe for allele discrimination in dark blue. Red boxes indicate the positions of exon 1 with the translation start site, and of exons 2 and 3 that become disrupted. SacI sites and bidirectional arrows between them denote restriction fragment positions and lengths used in Supplemental Figure 2B. Arrowheads indicate the positions of primers used in Supplemental Figure 2C.

(B) Genotype determination by Southern blot analysis of WT (+/+), heterozygous (+/-) and homozygous (-/-) ΔRFWD3-DT40 cells. SacI-digested genomic DNA was hybridized with the probe shown in Supplemental Figure 2A.

(C) RT-PCR analysis of chicken RFWD3 mRNA expression in control and ΔRFWD3-DT40 cells using the primers shown in Supplemental Figure 2A.

(D) Relative cell growth of WT-DT40, ΔRFWD3-DT40 and ΔFANCD2-DT40 cells over a period of four days.

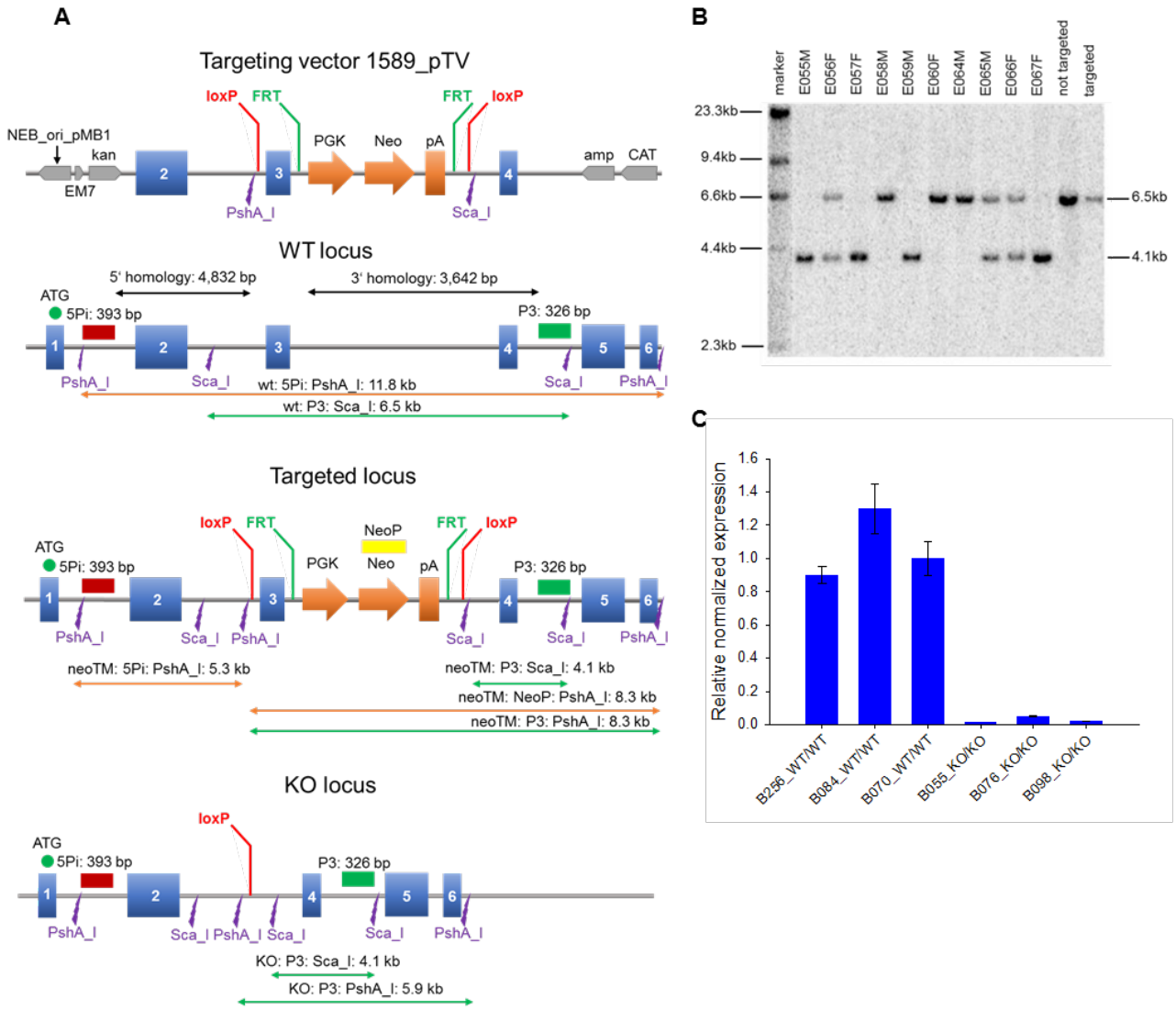
(E) Structure of the SCneo construct and expected HR repair products revealing STGC (short-tract gene conversion) or LTGC/SCE (long-tract gene conversion/unequal sister chromatid exchange).

(F) Number of G418-resistant clones with STGC or LTGC/SCE events in WT-DT40 and ΔRFWD3-DT40 cells after transfection of I-SceI vector and selection by G418.

(G) A majority of MMC-induced FLAG-RFWD3 and FANCD2 foci in U2OS cells co-localized or were in close proximity.

(H) *RFWD3*-mutant HAP1 cells were transduced with the indicated siRNAs and analyzed by immunoblotting 24 h later. Silencing of FANCD2 resulted in the loss of corresponding protein, knock-down of BRCA2 in the reduction of RAD51 on chromatin.

(I) WT and *RFWD3*-mutant HAP1 cells were transduced with FANCD2 siRNA as above. Silencing resulted in similar loss of FANCD2 protein in cells of either genotype.



Supplemental Figure 3. Generation and characterization of *Rfwd3*$^{-/-}$ mice (related to Figure 5).

(A) Targeting strategy. The replacement vector contains a neo-cassette (orange) flanked by FRT sites (green) which, together with exon 3, are flanked by loxP sites (red). Using 5' and 3' homology arms the vector was used to target the *Rfwd3* locus of mouse ES. After selection with G418, exon 3 and the neo-cassette were removed by Cre-mediated recombination. The FRT sites for conditional KO were not used. Blue boxes denote exons. P3 (green) 5Pi (red) and NeoP (yellow) represent Southern hybridization probes of specified sizes. PshAI and ScaI designate restriction sites (purple bolts). Bidirectional orange and green arrows display restriction fragments of indicated sizes detected by corresponding probes.

(B) Southern blot analysis of *Rfwd3*$^{-/-}$, *Rfwd3*$^{+/-}$ and *Rfwd3*$^{+/+}$ mice. ScaI-digested genomic DNA was hybridized with the probe P3. Expected sizes: WT 6.5 kb and KO 4.1 kb. E055, 057, 059 and 067 are *Rfwd3*$^{-/-}$; E056, 065 and 066 are *Rfwd3*$^{+/-}$; E058, 060 and 064 are *Rfwd3*$^{+/+}$.

(C) Partial map displaying qPCR primers and probe binding site on Rfwd3 cDNA for exon 3-5 RT assay (upper panel) to confirm loss of exon 3. Normalized relative Rfwd3 mRNA expression in samples from *Rfwd3*$^{-/-}$ and *Rfwd3*$^{+/+}$ mice. Data represent mean ± SD. *N*=3 (lower panel).

(D) Partial map displaying qPCR primers and probe binding site on Rfwd3 cDNA for exon 6-8 RT assay (upper panel) to show reduced transcript. Normalized relative Rfwd3 mRNA expression in samples from *Rfwd3*$^{-/-}$ and *Rfwd3*$^{+/+}$ mice. Data represent mean ± SD. *N*=3 (lower panel).

Supplemental Table 1. Statistical analysis of WES data (related to Results and Figure 1)

Number of total reads	113,599,566
Reads that passed QC	112,700,612
Reads on target	109,529,301 (97%)
Exome coverage	197x
Total number of detected variants	29,180
Reported SNPs (dbSNP Build 134)	24,675
Unknown variants in exons	3,962
Unknown variants at splice sites	666 (136 thereof at essential splice sites)
Total number of unkown variants in heterozygous condition	3,981 (including essential splice sites)
Insertions/deletions	577
Missense	2,211
Nonsense	100
Silent	982
Genes with ≥2 heterozygous unknown variants	385
Genes with ≥2 heterozygous variants, both predicted to be disease-causing	35
Genes involved in DNA repair with ≥2 heterozygous variants, both predicted to be disease-causing	3

Supplemental Table 2. Genes with ≥2 heterozygous sequence variants, both predicted to be disease-causing (related to Results and Figure 1)

LOC729737	KIAA1211
C1orf159	HGC6.3
SRRM1	CCDC146
HORMAD1	ZNF696
C2orf71/RP54	ANKRD18B
PLEKHH2	ANKRD20A2
LOC400986/ANKRD36C	ANKRD20A1
LOC100287144/USP17L10	ANKRD2
C10orf76	ZNF408/RP72
LOC100507338/PFM8	USP15
FLYWCH1	**RFWD3**
ROGDI	KIAA0664/CLUH
LOC400499	CBX4
ARMC5	AATK
ZNF527	PRR12
ZNF784	**MCM3AP**
ZNF335/MCPH10	MN1
ESX1	

Genes involved in DNA repair are marked bold. RFWD3 is highlighted in red.

Supplemental Table 3. Cell lines and animals used in this study (related to Methods)

Cell line or animal	**Source or Reference**
1143, 1143 + WT-RFWD3	This study
1143 + mock, 1143 + RFWD3-I639K	This study
FA-B, -D1, -P, -Q and non-FA control fibroblast lines	This study
U-2 OS (U2OS) parental	Sigma-Aldrich, 92022711
U2OS CR21F5	This study
HAP1 parental	Horizon Discovery, C859
HAP1 RFWD3 knockout	Horizon Discovery, HZGHC002532c010
HAP1 RFWD3 knockout + RFWD3 WT	This study
DT40 parental	Riken BRC, RCB1464
DT40 ΔFANCD2	Mol Cell Biol, 21: 2858-2866 (2001)
DT40 ΔXRCC3	Mol Cell Biol, 25: 34-43 (2005)
DT40 ΔRFWD3	This study
DT40 ΔRFWD3 + RFWD3 WT	This study
DT40 ΔRFWD3 + RFWD3 I615K	This study
DT40 ΔRFWD3 + RFWD3 C267A	This study
DT40 Scneo	J Biol Chem, 276: 44413-44418 (2001)
DT40 Scneo ΔRFWD3 + RFWD3 WT	This study
DT40 Scneo ΔRFWD3 + RFWD3 I615K	This study
DT40 Scneo ΔRFWD3 + RFWD3 C267A	This study
Rfwd3$^{-/-}$, *Rfwd3*$^{+/-}$ and *Rfwd3*$^{+/+}$ C57BL/6 mice	Ozgene

Supplemental Table 4. Human genomic primer sequences (related to Methods)

Name	Sequence	Used for
RFWD3_Ex_2_F	AAGTCCATTACCAAACACTTCTGACTTAAGTA	c.204_205dupCC validation
RFWD3_Ex_2_R	CATAATTTCTAGTACAGCAATGATCACAGACT	c.204_205dupCC validation
RFWD3_Ex_3_F	AGCATTTAATGGCCTGGAGATGGGTTGA	Sanger sequencing
RFWD3_Ex_3_R	TCTACATAATTATGGCCAGTGAAGGCTCTATA	Sanger sequencing
RFWD3_Ex_4_F	CTGCTAGCTTCTCATACCTGACATATCTTAT	Sanger sequencing
RFWD3_Ex_4_R	ATATTCAGGTTGGGCATGGTGGCTC	Sanger sequencing
RFWD3_Ex_5-6_F	TACAAGTGTTCTGTAGCCCTTTTGATTGTA	Sanger sequencing
RFWD3_Ex_5-6_R	AATTATAAATAACAGTGTAAGACAGACTGCCAATG	Sanger sequencing
RFWD3_Ex_7_F	TACAGCAAAGAACGTGCATCATAGCATGAA	Sanger sequencing
RFWD3_Ex_7_R	TATAGTAGACCTCATCATAAAGTGTGTA	Sanger sequencing
RFWD3_Ex_8_F	CAGTTCTCTAGGATTCATGTTAACACTTTCATA	Sanger sequencing
RFWD3_Ex_8_R	ATCTTTGCAGCACCCTGGGGTACTT	Sanger sequencing
RFWD3_Ex_9_F	TGACCAGCTGGTTCCCAAGGACTTATA	Sanger sequencing
RFWD3_Ex_9_R	AACAAAAAACAGAGCTAAGGTCATCATAAC	Sanger sequencing
RFWD3_Ex_10_F	AGTGTGTTCTCAGTAAGGATTAATGATCA	Sanger sequencing
RFWD3_Ex_10_R	AACCTGGCTATACTTGTACCAGAACTCTC	Sanger sequencing
RFWD3_Ex_11_F	TCTGGAGCCGTCCGAGTTTTGGA	c.1916 T>A validation
RFWD3_Ex_11_R	ACCTCGTTAGCCTGTGAGCGGTA	c.1916 T>A validation
RFWD3_Ex_12_F	TGCATTTATGGCTTTTCCATCCAACTAGGTT	Sanger sequencing
RFWD3_Ex_12_R	GACTCATGGAAACCAAGCAATCTCACATA	Sanger sequencing
RFWD3_Ex_13_F	GTCAGGTGTGCTGACTTTGAACAACCTT	Sanger sequencing
RFWD3_Ex_13_R	TCCTAGAATCATACTAATGCAAACAAACCATGAT	Sanger sequencing
RFWD3_Ex_c2_F	GGCCGAGGTAACTACCGAGTCTT	cDNA Sanger sequencing
RFWD3_Ex_c2_R	AAGTCAGGCTGGGTCCTGCTCA	cDNA Sanger sequencing
RFWD3_Ex_c11_F	GGCTAACTACATCTATGCTGGACTGG	cDNA Sanger sequencing
RFWD3_Ex_c11_R	TTTTGGTCAATAGTTTGCAAGTAGGTCCT	cDNA Sanger sequencing

Supplemental Table 5. Primer sequences used for RFWD3 cloning and analysis (related to Methods)

Name	**Sequence**	**Used for**
Human_Puro_for	CGAGTTGAGCGGTTCCCGGCTGG	Transduction verification
Human_Puro_rev	GTTGATTGTTCCAGACGCGTCTAGGCACC	Transduction verification
Human_I639K_for	GCTGCCCTTGGAGCCAGGGGGCTGCAAAGACTTTCAG ACAGAGAACAGCTCCCGG	Mutagenesis
Human_I639K_rev	CCGGGAGCTGTTCTCTGTCTGAAAGTCTTTGCAGCCCC CTGGCTCCAAGGGCAGC	Mutagenesis
Human_pLVX-EF1a-IRES-puro_for	gagactagttctagaATGGCTCATGAAGCAATGGAATATGATG	Infusion cloning
Human_pLVX-EF1a-IRES-puro_rev	agaggggcgggatccTCACTCCCACTTATAGATGTGGACC	Infusion cloning
Human_pIRES_neo_for	ggcctgttaaccggtATGGCTCATGAAGCAATGGAATATGATG	Infusion cloning
Human_pIRES_neo_rev	ggatccgaattcgaaTCACTCCCACTTATAGATGTGGACC	Infusion cloning
DT40chicken_STGC_1	AACAGCTCTGGTAAGCTTGTCCAGATCGATG	Detection of STGC
DT40chicken_STGC_2	TGCTTTGCGTTCAAGCTTGGCTGCAGGTCG	Detection of STGC
DT40chicken_LTGC_1	GTGAGGAAGAGTTCTTGCAGCT	Detection of LTGC
DT40chicken_LTGC_2	CAGCGCCCGACCGAAAGGAGCGCACGACC	Detection of LTGC

Supplemental Table 6. Antibodies used in this study (related to Methods)

Name	Type of antibody	Company	Catalogue number	Used for	Dilution
Anti-FANCD2	Mouse monoclonal	Santa Cruz Biotechnology	sc-20022	Western blotting	1:500
Anti-FANCD2	Rabbit polyclonal	Novus	NB100-182	Western blotting Immunocytochemistry	1:5000 1:500
Anti-RAD51	Rabbit, polyclonal	abcam	ab63801	Immunofluorescence	1:800
Anti-RFWD3	Rabbit, polyclonal	abcam	ab138030	Western blotting	1:1000
Anti-Tubulin	Mouse, monoclonal	abcam	ab44928	Western blotting	1:1000
Anti-Tubulin	Mouse monoclonal	Sigma-Aldrich	T5168	Western blotting	1:5000
Anti-GAPDH	Rabbit polyclonal	abcam	ab985-200	Western blotting	1:1000
Anti-p84	Mouse, monoclonal	abcam	ab487	Western blotting	1:2000
Anti-Histone H3	Rabbit, polyclonal	abcam	ab1791	Western blotting	1:600
Anti-Histone H3	Mouse monoclonal	Active Motif	39763	Western blotting	1:2000
Anti-RAD50	Mouse, monoclonal	GeneTex	GTX70228	Western blotting	1:5000
Anti-Vinculin	Rabbit, monoclonal	abcam	ab129002	Western blotting	1:10000
Anti-RPA1	Rabbit, polyclonal	Bethyl Laboratories	A300-241A	Immunofluorescence	1:1000
Anti-RPA2	Rabbit, polyclonal	Bethyl Laboratories	A300-244A	Western blotting Immunofluorescence	1:5000 1:1000
Anti-RPA2	Mouse monoclonal	abcam	ab2175	Immunocytochemistry	1:500

Anti-GFP	Mouse monoclonal	MBL	M048-3	Western blotting Immunofluorescence	1:5000 1:500
Anti-FLAG	Mouse monoclonal	Sigma-Aldrich	F1804	Western blotting	1:2000
Anti-FLAG	Rabbit polyclonal	MBL	PM020	Immunocytochemistry	1:500
Anti-Rabbit IgG (H+L)	Goat polyclonal	Life Technologies	A11072	Immunofluorescence (Secondary antibody)	1:2000

Supplemental Table 7: Primer and probe sequences for mouse *Rfwd3*-targeting studies (related to Methods)

Name	Sequence	Used for
1589_41	CTAAAGGACAGTTAAGTGCATCTAGACC	PCR fragment 1
1589_51	CTTATGGTCTCTGACTCCAAGC	PCR fragment 1
1589_42	TAAGCATTGGTAAACCGGTGACACGTGTCATAACTTCGTATAGCATACATTATACGAAGT TATCCCAAGTGTTTGCCTGTGAGTGTAT	PCR fragment 2
1589_52	CTAAGGCGCGCCTCAGAATAAAAAACAGAAAGGGAATGCCATTACACAGG	PCR fragment 2
1589_43	CTAAAGTACTAATGATCCACCCAAGATAGTCACC	PCR fragment 3
1589_53	TAAGCATTGGTAAAAATAACAGTGCAAGGTTGGGTGG	PCR fragment 3
5Pi	CTTGTCGCTCGCTGTTTTAACTTTTATCACCGCAGCGGAACTGCGACCGAAGGATA TTTTCATTCTCCTCTTCCTGCGGGGAGGCGGGCGTGGGCAGACAGGTTAGGAAACT TAACTTTGGTTCCGCAGCTACTAACAGGGCGAGCCTGGATACGAACCCGGGTCTGAC TGAATCCCCGCGGGCTCCCGGCGACCTGTTTATTGGGGCAGCCGTCGCTCCCGCCTT CTGTGTTTATCGAAACCAAAGCTTAGCAAGGGTGGGGGGCAGAGGTGGAGTTCACGGT CTGAGGGCCGCTGACTTTTCGGGGCCATCGGACTAGCGTTTGTGTCACTTAAAGAAGG GGCTCATACGAGCACGAGCCCAGGATTTGGGGAACGTGTTTTGTTTTTGGA	Southern hybridization
P3	CCACCCAACCTTGCACTGTTATTTTTTGTTTCAGACAGTTTTGCTAGGCAGCCCAGACAG GCCTTGCTTTACTATCTGTTGTTTGCTGTCACAGGACACATTTAGTCTACTCCCTGTTGG GGTCCCCCTCCCCGCCTTTTAAACATTTTCTCGTATAAGTCAAGACTTTATATAGTTACCT TAACAAATTGTAGTGAGTATAGTCATGAGATTTATTTATAGCAGAGGTTGCCATATAAAA GGAAAGATGTTTTACTTTTGATGTGACCAACGAATGTGCCTTTGGAAATTTATGCAAGCT ATTGAAGCACCGATAAGCATGTCC	Southern hybridization
NeoP	GACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGC AGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCA GGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGT GCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGG GCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATG CAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAA ACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGAT CTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCG CGCATGCCCGACGGCGATGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATA TCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGC GGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGG	Southern hybridization

Prime Time Primer 1	CACCTTCCTCATCTTCCATAGC	RT-PCR Exon 3-5
Prime Time Primer 2	TGAAGCCAACATTCCAGAACA	RT-PCR Exon 3-5
Prime Time Probe A	/ 56-FAM / TCCCTAAAG / ZEN / AGTCTCCCCAGAAGCC / 31ABkFQ /	RT-PCR Exon 3-5
Prime Time Primer 1	CCGCACAACAAACTTTTCCA	RT-PCR Exon 6-8
Prime Time Primer 2	GTGACTTACTGAATGAGCAAATGC	RT-PCR Exon 6-8
Prime Time Probe B	/ 56-FAM / AATAAGGAC / ZEN / CTGAAGCTGGAGCCG / 31ABkFQ	RT-PCR Exon 6-8