

SUPPLEMENTARY INFORMATION (Ro and Rannala)

Methods

Primer sequences

STOPEGFP5: 5'-AAGAATTCGCCACCATGGTGGAGGAGTAGAGCAAGGGCGAGGAGCTGTTC-3'

3EGFP: 5'-GTGAATTCTTACTTGTACAGCTC-3'

EGFPMIDDLE5: 5'-GAGGGCGACACCCTGGTGAACC-3'

Generation of revertant forms of the stop-EGFP gene

Three possible revertant forms of the stop-EGFP gene, each containing AAG (encoding lysine), TTG (encoding leucine), or TGG (encoding tryptophan) at the site of the premature stop codon, were generated by PCR using pCX-stop-EGFP (plasmid containing the stop-EGFP gene) as a template:

5'-AAGAATTCGCCACCATGGTGGAGGAGA**A**AGAGCAAGGGCGAGGAGCTGTTC-3',

5'-AAGAATTCGCCACCATGGTGGAGGAG**TT**GAGCAAGGGCGAGGAGCTGTTC-3',

or 5'-AAGAATTCGCCACCATGGTGGAGGAG**TGG**AGCAAGGGCGAGGAGCTGTTC-3'

as a forward primer, and 3EGFP as a backward primer. Each PCR product was purified and subsequently used to replace the stop-EGFP gene within pCX-stop-EGFP. The resulting constructs are referred to as pCX-Lys-EGFP, pCX-Leu-EGFP and pCX-Trp-EGFP, respectively. Sequence changes were confirmed by DNA sequencing.

Genomic PCR and Southern blot analysis

Tail tip biopsies from mice were used for extraction of genomic DNA. A genomic PCR was performed using the following primers:

5'-GCAAAGAATTCGCCACCATG-3'

5'-CCGTTTACGTCGCCGTCAG-3'

These primers are designed to generate PCR products of 100 bp from the stop-EGFP gene and 91 bp from the EBFP gene (see Fig S1). For the separation of the two bands (i.e., 100 bp and 91 bp), 4% low melting agarose was used. In Southern blot analysis, 10 µg of genomic DNA per sample was digested with *Pst*I and probed with the *Eco*RI fragment of pCX-stop-EGFP.

Mutation assay in the kidney and the liver

At 5 months post-ENU administration, a stop-EGFP mouse was euthanized by cervical dislocation and perfused with 10 ml of saline followed by 10 ml of 4% paraformaldehyde. The liver and kidney were removed from the mouse and were stored in 4% paraformaldehyde at 4 °C with gentle agitation for 11 hours. After fixation, the organs were transferred to PBS with 1 mM MgCl₂ and stored at 4 °C overnight. The organs were sectioned into slices (200 µm in thickness) using a vibratome. Slices were mounted in glycerol mixed with PBS and then scanned using an inverted laser scanning confocal fluorescent microscope (Zeiss LSM 510) with a × 10 objective and an LP 520 emission filter (Zeiss). Green fluorescent mutants were imaged using the microscope with an Argon laser (488 nm) and an EGFP-specific bandpass filter.

DAPI staining and colocalization of nuclei

Several slices containing EGFP signals were selected and used to verify colocalization of DAPI-stained nuclei and bright EGFP signals within mutant cells. Slices were incubated for 30 minutes at room temperature in a PBS solution containing 1 µg/ml of DAPI. Slices were briefly washed with PBS, and then dehydrated by immersion in 30, 50, 60, 70, 90 and 100% ethanol, serially for 5 minutes at each step. Slices were mounted in methyl salicylate and imaged using a × 25 objective. DAPI-stained DNA was activated with a two-photon laser of 760 nm.

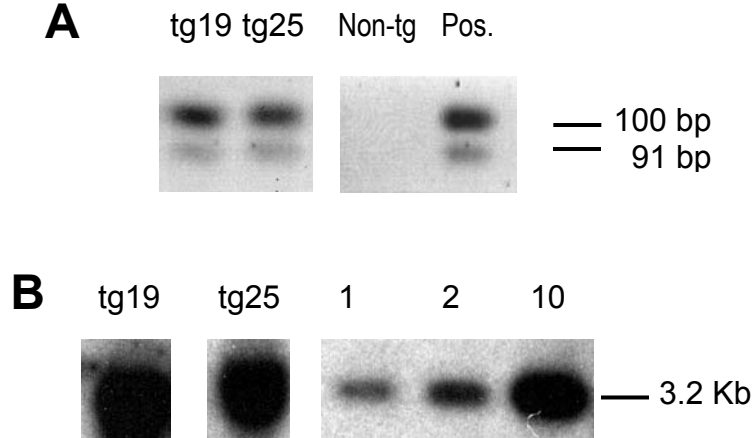


Figure S1 Genomic PCR and Southern blot analysis. **(A)** A PCR analysis of genomic DNA from two transgenic founders (tg19 and tg25) was performed to determine whether they carry both the stop-EGFP gene and the wild-type EBFP gene. The primers used generate PCR products of 100 bp from the stop-EGFP gene and 91 bp from the EBFP gene. Non-transgenic mouse showed no bands at 91 and 100 bp sizes (Non-tg). As a positive control, pCX-stop-EGFP (plasmid containing the stop-EGFP gene) was mixed with pCX-EBFP (plasmid containing the EBFP gene) and then used for the PCR (Pos.). To make it easy to distinguish the stop-EGFP gene from the EBFP gene by PCR, six nucleotides were added right before the premature stop codon when the stop-EGFP gene was constructed. Thus, the stop-EGFP gene is 9 bp longer than the EBFP gene. **(B)** Southern blot analysis of genomic DNA from transgenic line 19 (tg19) and line 25 (tg25). The total copy numbers of the transgenes (the stop-EGFP gene and the EBFP gene together) in lines 19 and 25 are estimated to be more than 10. Copy numbers of 1, 2 and 10 were used as references.

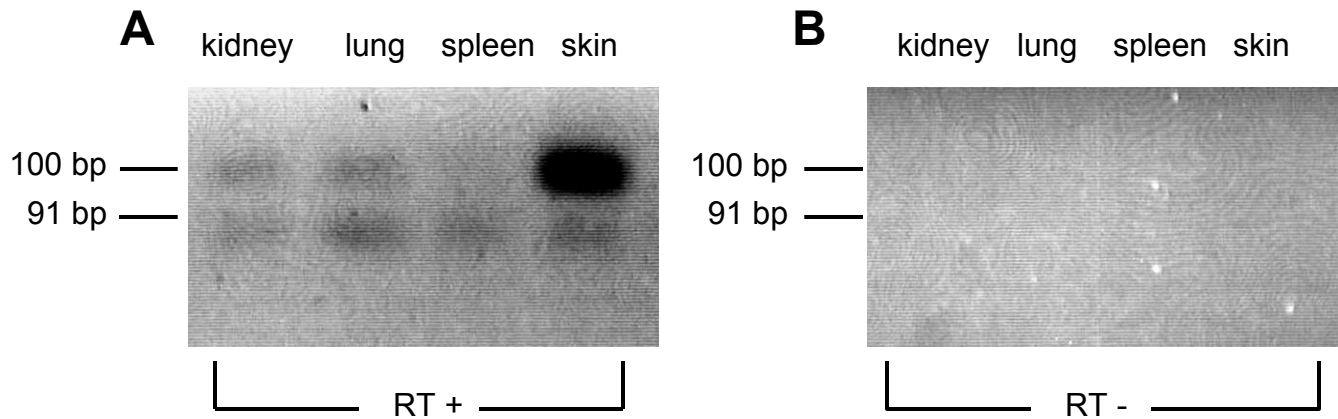


Figure S2 RT-PCR of mRNA collected from various tissues of the stop-EGFP mouse. **(A)** cDNA was synthesized from mRNA by reverse transcription and PCR was performed using the reverse transcribed cDNA. The primers used for the PCR generate PCR products of 100 bp from the stop-EGFP cDNA and 91 bp from the EBFP cDNA. The RT-PCR analysis shows that both the stop-EGFP gene and the EBFP gene are expressed in the kidney, lung, spleen and skin. Relative ratios of mRNA levels of the two genes seem to be varied depending on tissues. Further analysis will address this issue. **(B)** PCR was performed without reverse transcription. No bands were detected of either 100 bp or 91 bp size. This result demonstrates that the PCR bands shown in panel **A** were not generated from possibly contaminated genomic DNA.

Notes: mRNA transcript containing a premature stop codon is degraded by the mechanism called nonsense-mediated mRNA decay (Maquat, 2004; Singh & Lykke-Andersen, 2003). In a mammalian system, a stop codon located more than 50 to 55 nucleotides upstream of the last exon-exon junction of mRNA is recognized as a premature stop codon and the mRNA is led to rapid decay; mRNA transcript from a gene without an intron is not targeted for nonsense-mediated mRNA decay even if the mRNA contains a premature stop codon (Maquat & Li, 2001). Because the stop-EGFP gene does not contain an intron, mRNA transcribed from the stop-EGFP gene is not degraded, thus can be detected using RT-PCR.

Table S1 Summaries of *in vivo* imaging analyses of the dorsal epidermis of stop-EGFP mice treated with ENU. Approximately 2.5 cm × 2.5 cm area of the depilated dorsal skin of each mouse was scanned. In total, three green fluorescent epidermal cell lineages were identified by *in vivo* imaging of five ENU-treated stop-EGFP mice. No green fluorescent epidermal cells were detected in the dorsal skin of four untreated stop-EGFP mice, suggesting that the green fluorescent cell lineages observed were generated in adult mice exposed to ENU rather than arising because of the spontaneous mutation.

	Date of the first imaging (time after ENU administration)	Observation from the first imaging	Date of the second imaging (time after ENU administration)	Observation from the second imaging
Mouse #1	18 days	A green fluorescent cell lineage containing a single corneocyte	6 weeks	The cell lineage was not detected. (originated from a transit amplifying cell?)
Mouse #2	6 weeks	A green fluorescent cell lineage containing three adjacent corneocytes	Not performed	
Mouse #3	13 weeks	A green fluorescent cell lineage containing three adjacent corneocytes	16 weeks	The cell lineage was detected again.
Mouse #4	13 weeks	No green fluorescent cells detected	Not performed	
Mouse #5	13 weeks	No green fluorescent cells detected	Not performed	

References

Maquat LE, Li X (2001) Mammalian heat shock p70 and histone H4 transcripts, which derive from naturally intronless genes, are immune to nonsense-mediated decay. *RNA* **7**: 445-456

Maquat LE (2004) Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol* **5**: 89-99

Singh G, Lykke-Andersen J (2003) New insights into the formation of active nonsense-mediated decay complexes. *Trends Biochem Sci* **28**: 464-466