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Curr Protoc. Author manuscript; available in PMC 2026 January 01.

Published in final edited form as:

Curr Protoc. 2025 January ; 5(1): e70086. doi:10.1002/cpz1.70086.

Modeling Malignant Mesothelioma in Genetically Engineered Mice

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Abstract

Mesothelioma is a lethal cancer of the serosal lining of the body cavities. Risk factors include environmental and genetic factors. Asbestos exposure is considered the principal environmental risk factor, but other carcinogenic mineral fibers, such as erionite, also have a causal role. Pathogenic germline (heritable) mutations of specific genes, especially *BAP1*, are thought to predispose to mesothelioma in about 10% of cases. Somatic mutations and deletions of specific tumor suppressor genes, particularly *BAP1*, *CDKN2A/B*, and *NF2*, occur frequently in human mesothelioma, and asbestos-exposed mice with heterozygous deletions of any one of these genes have been shown to develop mesothelioma more often and at an accelerated rate than in control animals. Autochthonous mesothelioma mouse models, which are genetically engineered to carry multiple genetic lesions matching those observed in the human disease counterpart, closely resemble the disease phenotype and the extensive inflammatory responses that characterize human mesothelioma. Because autochthonous mice do not require asbestos and form tumors rapidly, these models are invaluable for assessing novel therapeutic strategies in an immunocompetent setting. The overlapping genetic, epigenetic, and immune environment of the tumors observed in these genetically engineered mouse models (GEMMs) with those found in human primary mesothelioma specimens supports the clinical relevance of these preclinical models. This review presents protocols for studies of asbestos-induced mesothelioma in GEMMs and non-carcinogenic conditional knockout models of mesothelioma, including an example of a preclinical application. These models are invaluable for understanding the biological underpinnings of mesothelioma and for testing new therapeutics and chemoprevention or interception agents.

Basic Protocol 1: Generation of a GEMM with a Germline *Bap1* Knockout Allele

Basic Protocol 2: Generation of GEMMs with Germline *Bap1* Knock-in Alleles

Basic Protocol 3: Asbestos Carcinogenicity Investigations with GEMMs

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CONFLICT OF INTEREST STATEMENT

J.R. Testa has a patent for *BAP1* mutation testing and has also provided legal consultation regarding genetic aspects of mesothelioma. The remaining authors have no potential conflicts of interest to declare regarding the publication of this work.

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Basic Protocol 4: Preclinical Chemoprevention and Chemotherapy Studies Using a GEMM with Asbestos-Induced Mesothelioma

Basic Protocol 5: Generation of a GEMM with Conditional Knockout of *Bap1*

Basic Protocol 6: Generation of a Conditional Knockout Model of Mesothelioma

Keywords

Mesothelioma; asbestos carcinogenicity; intrapleural tumors; intraperitoneal tumors; conditional knockout mice

INTRODUCTION

Mesothelioma is an incurable cancer of the serosal linings of the chest, abdomen, and tunica vaginalis causally linked to asbestos exposure. Both environmental and genetic risk factors influence disease susceptibility (Carbone et al., 2019). Asbestos-induced inflammation and DNA damage play critical roles in mesothelioma pathogenesis, with a latency period of several decades. Inactivating somatic mutations and deletions of the tumor suppressor genes (TSGs) *BAP1*, *CDKN2A/B*, and *NF2* are the most frequent genetic lesions in human malignant pleural mesothelioma (MPM). Alterations of these three TSGs are frequently seen in various combinations in an MPM (Bott et al., 2011; Bueno et al., 2016). The idea that inactivation of these specific TSGs is so prevalent implies that the signaling pathways regulated by these genes are fundamental to the development of MPM (Testa & Berns, 2020).

Mesothelioma patients, MPM in particular, are often surgically inoperable and refractory to standard therapy. Immunotherapies have become a standard treatment for MPM patients. Still, the durability of most therapeutic responses remains short and ultimately results in relapse (Fennell et al., 2022). Consequently, there is an urgent need for innovative approaches to reduce the overall incidence of mesothelioma and improve therapies. *In vivo* models are required to investigate mesothelioma disease pathogenesis to aid in this effort and provide faithful preclinical models to identify novel therapies that might advance toward clinical trials (Testa & Berns, 2020). There is also hope that specific drugs or natural substances could be used to prevent cancer in individuals who are at high risk of developing the disease as a means of early intervention (chemoprevention) or by intervening at an early stage in the tumorigenic process before a full-blown incurable tumor develops (cancer interception) (Blackburn, 2011).

There are several recent reviews of preclinical mouse models of mesothelioma and their uses in studying disease pathogenesis and discovering and developing mesothelioma therapies (Blanquart et al., 2020; Testa & Berns, 2020; Seastedt et al., 2021; Shamseddin et al., 2021). In addition to genetically engineered mouse models (GEMMs), a MexTAg transgenic mouse model utilizes the mesothelin gene promoter to express SV40 large T antigen specifically in the mesothelial lining (Robinson et al., 2006, 2011). Although this model does not have any of the genetic hallmarks attributed to the human disease, gene expression profiling of mesotheliomas from MexTAg mice has been shown to exhibit a concordant

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set of deregulated genes compared to normal mesothelial cells that overlapped with the deregulated genes between human mesotheliomas and mesothelial cells (Robinson et al., 2015). Patient-derived xenograft (PDX) models, in which tumor fragments are grafted into immunodeficient recipient mice, more closely recapitulate the human disease and typically maintain their human stromal features for multiple passages. PDX models permit studies of inter- and intra-tumor heterogeneity and attributes dictated by the distinct genetic features of individual tumors (Nabavi et al., 2018). In contrast, their propagation must be performed in immunodeficient mice unless expensive and technically demanding humanized host models are used. Orthotopic intrapleural models have also been reported. For example, Servais and colleagues described a murine immunocompetent orthotopic model of pleural cancer that recapitulates the human pleural setting and microenvironment and can be used in combination with bioluminescent imaging to monitor tumor burden and allow for studies of inflammation on tumor progression (Servais et al., 2011). However, for preclinical investigations of therapies targeting human antigens, immunodeficient models are required to perform studies on xenografted human cancer cell lines. The necessity to use immunodeficient mice as a host for such graft experiments complicates the assessment of immunomodulating effects.

While no single model is likely to have every desirable feature, the malignancy developing in the model should mimic at least several of the most relevant features of human mesothelioma, such as its pathology, its genetic driver alterations, its gene expression profiles, and the inflammatory phenotype that is characteristic of the disease (Testa & Berns, 2020). Ideally, the model should also exhibit a reproducible and short tumor latency period to allow for preclinical intervention studies. Given the ability of asbestos to induce inflammation that plays a role in mesothelioma pathogenesis (Wang et al., 2004; Stadlmann et al., 2006; Kadariya et al., 2016b), asbestos carcinogenicity models are also invaluable for assessing gene-environment interactions in GEMMs. Here, we present GEMM protocols used in our laboratory to investigate gene-environment interactions and gene-gene cooperativity in mesothelioma susceptibility and tumor progression.

BASIC PROTOCOL 1: GENERATION OF A GEMM WITH A GERMLINE *Bap1* KNOCKOUT ALLELE

This protocol describes how a GEMM with a heterozygous deletion of a tumor suppressor gene, e.g., *Bap1*, implicated in human mesothelioma pathogenesis, is generated for subsequent asbestos carcinogenicity studies. Interested investigators could use a similar approach to develop a heterozygous deletion of other tumor suppressor genes in mesothelioma as new players are implicated in future years.

To assess the susceptibility of a heterozygous mutant GEMM, e.g., $Bap1^{+/-}$ mice, to the carcinogenic effects of asbestos, $Bap1^{+/-}$ and $Bap1^{+/+}$ (wild-type, WT) littermates were chronically injected intraperitoneally (i.p.) with asbestos per our usual method (Altomare et al., 2005; Altomare et al., 2011; Menges et al., 2014; Xu et al., 2014; Kadariya et al., 2016a, 2016b), as described in detail below. Although direct delivery of asbestos into the peritoneal cavity is an unnatural route of exposure, the pathology and growth pattern of the

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tumors are similar to human diffuse mesotheliomas (Marsella et al., 1997; Altomare et al., 2005). Furthermore, asbestos administration by inhalation or intratracheal injection requires special equipment, and this approach induces relatively few mesotheliomas that necessitate a much longer time to form. Using the i.p. protocol described here, asbestos-exposed mice with germline heterozygous mutations of either *Bap1*, *Nf2*, or *Cdkn2a* alone develop mesothelioma with median survivals of 8–10 months (Altomare et al., 2005; Altomare et al., 2011; Kadariya et al., 2016a; Xu et al., 2014), whereas asbestos-exposed WT littermates showed delayed onset and fewer tumors, with median survivals of 12–14 months.

Zinc finger nuclease (ZFN) technology (Meyer et al., 2010; Cui et al., 2011) can be used to generate heterozygous *Bap1* mice with knockout or knock-in alleles in an FVB genetic background. The specific method is similar to that previously reported for ZFN-mediated gene targeting in mouse embryos (Mookerjee-Basu et al., 2019). Three different heterozygous *Bap1* GEMMs are described here using ZFN technology: *Bap1* knockout ($Bap1^{+/-}$), *Bap1* "Lou" knock-in ($Bap1^{+/L}$), and *Bap1* "Wis" knock-in ($Bap1^{+/W}$). The *Bap1* knockout ($Bap1^{+/-}$) is described in Basic Protocol 1.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and follow officially approved procedures for the care and use of laboratory animals. Note also that while the materials and methods below have been reported already in brief (Xu et al., 2014; Kadariya et al., 2016a, 2016b; Kukuyan et al., 2019), they are presented in the "present tense" here as detailed protocols.

Materials

Zinc finger expression plasmids (Sigma-Aldrich)

NEBuffer 4, 10X (New England Biolabs, cat. no. B7004S)

100X BSA

*Xba*I (New England Biolabs, cat. no. R0145S)

Pipettes and tips

Nuclease free H_2O

UltraPure DNase/RNase-Free distilled water (ThermoFisher, cat. no. 10977023)

MessageMAX T7 ARCA-Capped Message Transcription Kit (Cellscript, cat. no. C-MMA60710)

Poly(A) Tailing Kit (ThermoFisher Scientific, cat. no. AM1350)

MEGAClear Transcription Clean-Up Kit (ThermoFisher, cat. no. AM1908)

100 mM DTT

DEPC-RNase-free Treated Water (ThermoFisher, cat. no. AM9915G)

PPE (disposable gown, head cover, gloves, safety glasses, N95 mask, shoe covers)

FVB/N F1 virgin females

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Pregnant mare’s serum gonadotropin (PMSG; stock solution: 50 IU/ml) (Prospec HOR-272)

Human chorionic gonadotropin (hCG) (Millipore Sigma C-1063)

ALPHA-dri bedding

Natural Crinklets (SAFE Enrichment)

Nestlets (Ancare, cat. no. NES3600)

Exel International Insulin Syringes (Fisher Scientific, cat. no.14–841-31)

Wescodyne/alcohol

Stereomicroscope

M2 culture medium (Millipore Sigma M-7167)

T10E0.1 Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0), (Biosolution, cat. no. OT010)

Nikon inverted microscope with DIC optics, Leitz micromanipulators, and cooling stage

Ketamine-acepromazine-xylazine cocktail (2.5 mg ketamine, 0.05 acepromazine, 0.25 xylazine

per ml); stock solution can be stored at 4°C for up to 3 months

Glass pipettes (500-micron inner diameter)

Mouse surgical set (autoclave before use)

Absorbable suture (5–0)

Stainless steel wound clips

Autoclave

Warming tray

Puregene Tissue Kit (Qiagen, cat. no. 158063)

PfuTurbo DNA Polymerase (Agilent Technologies, cat. no. 600250)

dNTP Mixture (Takara, cat. no. 4030)

QIAquick Gel DNA Extraction kit (Qiagen, cat. no. 28706)

DNA sequencing (Genewiz from Azenta Life Sciences)

Direct Mouse Genotyping kit (APExBIO, cat. no. K1025)

GoTaq Green Master Mix (Promega, cat. no. M712)

Forward and Reverse primers for genotyping (Integrated DNA Technologies IDT)

rCutSmart Buffer, 10X containing recombinant albumin (New England Biolabs, cat. no. B6004S)

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*Hpa*II restriction enzyme (New England Biolabs, cat. no. R0171)

*Hpy188*III restriction enzyme (New England Biolabs, cat. no. R0622)

Thermal cycler (PCR machine)

Water bath (37°C)

CD-1 mice (males and pseudopregnant females)

PR1MA Agarose (Midsci, cat. no. MIDSCI-500)

Tris-Borate-EDTA buffer, 10X (Santa Cruz Biotechnology, cat. no. sc-296650)

PR1MA SmartCheck DNA Ladders (MidSci, cat. no. PR4010)

Ethidium Bromide Solution 10 mg/mL (ThermoFisher, cat. no 17898)

Generation of a *Bap1* GEMM with a Knockout Allele—Design custom ZFNs targeting a *Bap1* sequence with an intronic cutting site targeting *Bap1* intron 5. Use Sigma-Aldrich's custom service to create ZFN expression plasmids. This service includes validation of the targets in mammalian Neuro-2a cells. Note that the sequence for the binding and cutting sites of the ZFNs is TGCTCCCCAGTAGTCACTGATGGCTGTGCACG, with the spacer between the binding sites underlined.

1. Linearize the ZFN expression plasmids cutting at the *Xba*I site located at the 3′ end of the *Fok*I ORF.
2. Set up the following digestion for *Xba*I digestion and incubate at 37°C for 2 h, followed by post-digestion purification.

ZFN plasmid (20 ηg)	12 µl
NEBuffer 4 (10X)	10 µl
100X BSA	1 µl
*Xba*I (20 U/µl)	8 µl
Nuclease free H_2O	69 µl
Total volume	100 µl

3. In vitro transcription: Prepare 5′ capped and 3′ poly(A)-tailed messenger RNAs using MessageMAX T7 ARCA-Capped Message Transcription (Cellscript) and Poly(A) Tailing (ThermoFisher) Kits and purify RNAs using a MEGAClear Transcription Clean-Up Kit (ThemoFisher), followed by incubation at 37°C for 1 h.

Plasmid DNA	2 µl
10X transcription Buffer	4 µl

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MessageMAX T7 ARCA Cap/NTP Premix	16 µl
100 mM DTT	4 µl
RNase free water	10 µl
MessageMAX T7 enzyme solution	4 µl
Total Volume	40 µl

Below is a workflow diagram of the procedures for isolating fertilized eggs, injecting pronuclear fluid, and implanting microinjected embryos (Fig. 1).

Fertilized Egg Isolation

4. Inject donor FVB/N F1 virgin females (4–5 weeks of age) i.p. with PMSG (pregnant mare's serum gonadotropin 5 IU/0.1 ml) on day 1 at 4 pm and hCG (human chorionic gonadotropin 5 IU/0.1 ml) i.p. on day 3 at 3 pm, and immediately place with stud males (2–6 months of age; 1 male per female). The animals are housed on ALPHA-dri bedding with bedding enrichment such as Natural Crinklets or Nestlets to promote a favorable environment for mating.

5. On day 4, sacrifice the pregnant donor females by cervical dislocation. The abdomen is wiped with Wescodyne, and a mid-ventral, longitudinal incision is made through the skin and body wall. Under a stereomicroscope, remove the oviducts and place them in sterile M2 culture medium, where the fertilized eggs are flushed from the ampulla with 100 µl of M2 medium.

Pronuclear injection

6. Under aseptic conditions, prepare a master mix containing DNA construct (5–10 ng/µl) and ZFN mRNAs (20–40 ng/µl) in injection buffer (T10-E0.1 buffer).

7. Deliver an estimated 0.5–1 pL of the master mix into one (of two) pronuclei of each fertilized single-cell mouse embryo by microinjection using an inverted microscope (Nikon) equipped with DIC optics, Leitz micromanipulators and a cooling stage.

Implantation of microinjected embryos

8. Pseudopregnant females are obtained by mating 4–6-month-old CD-1 females to vasectomized CD-1 males overnight and then selecting for plugged CD-1 females.

9. Anesthetize the pseudopregnant recipient female mice (day 0.5 pc from sterile mating with vasectomized males) by i.p. injection of ketamine-acepromazine-xylazine cocktail at a dose of 100 µl per 25 g mouse.

10. Autoclave surgical instruments before surgery. Prepare the incision site of the recipient mice with betadine/alcohol and shave. Use a marker pen to indicate the location of the longitudinal incision that is then made through the skin at a point approximately level with the kidneys. Make a similar incision through the body wall (Fig. 2).

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11. Expose one oviduct. Use a stereomicroscope to guide the transplant of the injected embryos into the ampulla of the oviduct using a 500-µ (inner diameter) glass pipette (Fig. 3A). Close the body wall with two sterile 5–0 absorbable sutures, and close the skin with stainless steel wound clips (Fig. 3B). All surgery is performed under aseptic conditions in a dedicated surgical area. All surgical supplies and solutions are single-use and sterile.

12. During recovery from surgery, house the mice on a warming tray until they display normal movement (usually for <4 h). Remove the stainless steel wound clips 7–14 days after surgery. Surgical staff should monitor the rodents for pain/distress and infection at least once daily.

Genotyping of heterozygous *Bap1* knockout mice

13. Within 7–10 days (check the specific number of days permitted by consulting your institution's IACUC), clip the tails of the pups derived from the pseudopregnant mice. Tail DNA samples are obtained by digestion and purification using a Gentra Puregene tissue DNA extraction kit (Qiagen).

14. Genotype the tails from all pups by amplifying DNAs using forward primer, 5'-AGGCTTTGCTGCTAAATGAGA-3'; and reverse primer, 5'-CCCTGAGACCCAGAAAATCA-3'. Thermal PCR cycling conditions are 95°C (5 minutes), followed by 35 cycles at 95°C for denaturation (30 seconds), 58°C for annealing (30 seconds), and 72°C for extension (45 seconds), with a final extension at 72°C (10 minutes).

10X PfuTurbo buffer	2.5 µl
dNTPs	2.5 µl
Forward primer 10 µm	1.0 µl
Reverse primer 10 µm	1.0 µl
PfuTurbo DNA polymerase	0.5 µl
Nuclease free water	16.5 µl
DNA	1.0 µl
Total Volume	25.0 µl

15. Resolve the PCR products on a 1% agarose gel for 30 minutes. Extract and purify the DNA from the gel using a QIAquick Gel DNA extraction kit (Qiagen) for subsequent DNA sequence analysis.

16. Send the PCR amplified products for sequencing to verify correct targeting. Sequencing can be done in-house or commercially, e.g., Genewiz from Azenta Life Sciences.

17. After identifying positive founder pups using the above mutation-specific primers, these animals are mated to FVB/N mice to generate stable heritable knockout lines. Genotyping is performed by extracting DNA from the mouse

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tails of the offspring using a Direct Mouse Genotyping Kit. PCR is performed using a GoTaq Green Master Mix (Promega).

2X GoTaq Green Master Mix	10 µl
forward primer 10 µm	1 µl
reverse primer 10µm	1 µl
Nuclease free Water	7 µl
DNA	1 µl
Total Volume	20 µl

Thermal PCR cycling conditions are 95°C (5 minutes), followed by 29 cycles at 94°C (30 seconds), 60°C for annealing (30 seconds), and 72°C for extension (35 seconds), with a final extension at 72°C (10 minutes). Representative genotyping of mice with a germline heterozygous *Bap1* knockout allele is shown in Fig. 4.

NOTE: The net effect of the *Bap1* deletion of exons 6 and 7 deletion is similar, but not identical, to that observed in a human BAP1 Tumor Predisposition Syndrome (BAP1-TPS) family having an intron 6 splice site mutation in *BAP1* that results in loss of exon 7 (Testa et al., 2011).

BASIC PROTOCOL 2: GENERATION OF GEMMS WITH *Bap1* KNOCK-IN ALLELES

As an alternative to generating a mouse with a germline *Bap1* knockout allele, the investigator may create a mouse model with a knock-in allele of interest. For example, we created two mouse knock-in models with inactivating *Bap1* mutations ($Bap1^{+/W}$ and $Bap1^{+/L}$) identical to those observed in the first two reported mesothelioma families with germline *BAP1* mutations, i.e., families Wis (from Wisconsin) and Lou (from Louisiana) (Testa et al. 2011). The investigator may use the same ZFN protocol described above but different custom ZFNs. Fertilized egg isolation, pronuclear injection, and implantation of microinjected embryos are performed as described above. The *Bap1* Lou knock-in mutation in exon 16 creates the identical stop codon seen in human family L. The *Bap1* Wis knock-in mutation generates an mRNA that differs from that observed in human family W (due to sequence divergence in exon 7 in human and mouse genomes). However, *Bap1* Wis-encoded mRNAs lose at least a portion of exon 7, and the net result is the same as the human W mutation, i.e., a premature truncation of the predicted gene product. A schematic diagram showing the cutting sites of the ZFNs and relevant portions of the respective *Bap1* knock-in alleles for the $Bap1^{+/W}$ and $Bap1^{+/L}$ mutant models is shown in Fig. 5. For the $Bap1^{+/W}$ knock-in model, the custom ZFNs targeted the same sequence described above with a cutting site of ZFNs targeting *Bap1* intron 5. For the $Bap1^{+/L}$ knock-in model, we designed custom ZFNs targeting a *Bap1* sequence with a cutting site of ZFNs targeting *Bap1* exon 16. Sigma-Aldrich validated all these targets in mammalian cells. After the knock-in founder lines are identified, the following steps are used for genotyping the offspring.

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Materials

The materials are identical to those in Basic Protocol 1, except that the ZFN expression plasmids (Sigma-Aldrich) and genotyping primers and conditions differ.

Genotyping *Bap1*$^{+/W}$ mice

1. A Direct Mouse Genotyping kit (APExBIO) is used to extract DNA for genotyping.
2. Amplify the DNA by PCR amplification using the forward primer using the forward primer mBap1 E6–2F: 5'-ATTTTTAGAGCAGCAAAGGATATGCAA-3' and the reverse primer mBap1 E7–2R:5'- TCCATCCAATTCAAAGAGCCT-3'. Thermal PCR cycling conditions used are 95°C (5 minutes), followed by 35 cycles at 95°C for denaturation (30 seconds), 58°C for annealing (30 seconds), and 72°C for extension (45 seconds), with final extension at 72°C (10 minutes).

2X GoTaq Green Master Mix	10 µl
mBap1 E6–2F primer 10 µm	1 µl
mBap1 E7–2R primer 10 µm	1 µl
Nuclease free Water	7 µl
DNA	1 µl
Total Volume	20 µl

3. Subject the PCR product to enzymatic digestion using the restriction enzyme *Hpa*II to identify the knock-in alleles.

Nuclease free water	12.5 µl
10X rCutSmart Buffer	2.0 µl
*Hpa*II restriction enzyme	0.5 µl
PCR product	5.0 µl
Total volume	20.0 µl

4. Incubate the reaction at 37°C for 1 h and resolve undigested and digested PCR products side by side on 2% agarose gels for 30 minutes. Fig. 6 shows an example of genotyping heterozygous *Bap1*$^{+/W}$ knock-in mice.

Genotyping *Bap1*$^{+/L}$ mice

1. Use a Direct Mouse Genotyping Kit (APExBIO) to extract DNA from individual mouse tails for genotyping.
2. Amplify the DNA by PCR using the forward primer LouDonor ZFN-2F. 5'-AGGTGGGTGACCCCTCTACT-3' and the reverse primer LouDonor ZFN-2R 5'-CACTAGGTTGGCCAGCATTC-3'. The thermal cycling conditions are

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denaturation at 95°C (5 minutes), followed by 30 cycles of denaturation at 95°C (30 seconds), 60°C for annealing (30 seconds), 72°C for extension (30 seconds), with a final extension at 72°C (10 minutes).

2X GoTaq Green Master Mix	10 µl
LouDonor ZFN-2F primer 10 µm	1 µl
LouDonor ZFN-2R primer 10 µm	1 µl
Nuclease free H_2O	7 µl
DNA	1 µl
Total volume	20 µl

3. To identify the knock-in allele, perform an enzymatic reaction on the PCR product using the restriction enzyme *Hpy*188III.

Nuclease free water	12.5 µl
10X rCutSmart Buffer	2.0 µl
*Hpy*188III	0.5 µl
PCR product	5.0 µl
Total volume	20.0 µl

4. Incubate the reaction at 37°C for 1 h and resolve undigested and digested PCR products side by side on a 2% agarose gel for 30 minutes. Fig. 7 presents a representative genotypic analysis of heterozygous $Bap1^{+/W}$ knock-in mice.

BASIC PROTOCOL 3: Asbestos Carcinogenicity Investigations With GEMMS

To assess whether mice with a germline heterozygous mutation of a tumor suppressor gene such as *Bap1* may have increased susceptibility to the carcinogenic effects of asbestos, it is necessary to expose a heterozygous *Bap1*-mutant cohort and a *Bap1* wild-type (WT) cohort to asbestos and then monitor for tumor development over a sufficient amount of time for the disease to develop. To have an adequate number of animals for robust statistical analyses, the investigator should consult with a biostatistician experienced in studies of mouse models before beginning an experiment. For tumor suppressor genes known to play a significant role in mesothelioma tumorigenesis, e.g., *Bap1*, *Nf2*, and *Cdkn2a*, 20–30 mice per group have been sufficient to detect highly significant differences in the incidence and time of onset of mesotheliomas between mutant and WT groups, even when low doses of chrysotile of crocidolite were used (Kadariya et al., 2024).

Regarding the asbestos doses used for asbestos carcinogenicity experiments, we have followed the original protocol of Marsella and colleagues, which we have referred to as our standard total dose of 3.2.mg per mouse, with individual doses given every 21

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days (Marsella et al., 1997). Lower crocidolite and chrysotile asbestos doses may also be used successfully, with individual doses modified accordingly (Kadariya et al., 2024). The original study by Marsella et al. (1997) was performed on *Tp53*-deficient mice; it consisted of 8 doses of 0.4 mg crocidolite given every 21 days when the initial asbestos-induced inflammation had subsided. Some of our more recent studies have been conducted with four doses of 0.8 mg every 21 days. Thus, the same total dose was used, but the time allotted to dosing was decreased. This has been necessary for investigations with compound heterozygous animals, e.g., $Nf2^{+/-};Cdkn2a^{+/-}$ mice, because some mice succumbed to tumors before all asbestos injections had been given.

Materials

PPE (disposable gown, head cover, safety glasses, N95 mask, shoe covers)

Biological Safety Cabinet

Chemical fume hood

Baking oven

Crocidolite Asbestos Analytical Standard Sample UICC (SPI Supplies, cat. no. 02704A-AB) or

Chrysotile B Canadian Asbestos Analytical Standard UICC (SPI Supplies, cat. no. 02740A-AB)

3M N95 Healthcare Particulate Respirators and Surgical Masks, 1860 Series (Fisher Scientific, cat. no. 18–992)

3M Rugged Comfort 6500 Series Half Facepiece Reusable Respirator (Fisher Scientific, cat. no. 19–096-912)

3M 6000 Series Cartridge/P100 Particulate Filter Combinations (Fisher Scientific, cat. no. 18–999-4552)

MicroGuard MP, Microporous Coverall with attached hood & boot, elastic wrist, elastic back, open ankle (Thomas Scientific, cat. no. 21A00P353–8019)

Face Shield (Fisher Scientific, cat. no 19–181-800C)

Ansell MICROFLE SafeGri SG-375 (Fisher Scientific, cat. no. 19–048-575D)

Reagent Alcohol (Denatured Alcohol), 70% (v/v) Ricca Chemical (Fisher Scientific, cat. no. 2546705)

Heterozygous *Bap1* mice

Econo-Cage Disposable System (irradiated) with air grommet (Lab Products, cat. no. LPI no. 72014-GI)

HBSS, no calcium, no magnesium, no phenol red (Fisher Scientific, cat. no. 14–175-079)

PYREX Reusable Media Storage Bottles (Fisher Scientific, 06–423B)

Aluminum foil (Reynolds)

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Magnetic stir bar

Magnetic stirrer

Ultrasonic cleaner (Branson 1510)

Earphone-type sound mufflers

BD General Use and PrecisionGlide Hypodermic Needles, 25 gauge(Fisher Scientific, cat. no. 14–826-49)

BD Slip Tip Sterile Syringes, BD 309659, 1 ml. (Fisher Scientific, cat. no. 14–823-434)

BD Alcohol Swabs (Fisher Scientific, cat. no. 1223K92)

Ziploc bags

Red sharps container

Autoclavable Biohazard Waste Bags 48 × 37 in (Fisher Scientific, cat. no.14–828-248)

Anti-Mesothelin polyclonal antibody (Thermo Fisher Scientific, cat. no. PA5–79698)

Anti-WT1 (Wilms Tumor protein) antibody [CAN-R9(IHC)-56–2] (Abcam, cat. no ab89901)

Anti-Cytokeratin 8 antibody, clone TROMA-1 (Sigma-Aldrich MABT329M)

GraphPad Prism Software (Dotmatics)

CAUTION*:* Asbestos is a known human category I carcinogen, and its use in a Laboratory Animal Facility must follow standard operating procedures (SOP) in accordance with Institutional Biosafety Committee policies and approval. All personnel should complete available institutional training sessions concerning biological safety and biological waste issues and read and fully adhere to SOP for safe handling of asbestos. Personnel must wear appropriate personal protective equipment (PPE), including a properly fitted respirator (minimum P2 filter, half face disposable particulate respirator; non-disposable particulate respirator with cartridge may be a better option, with silicon preferred versus rubber, as it fits better and not as hot). Disposable Nitrile gloves are recommended when handling asbestos. We recommend double-gloving. Safety glasses with side shields or chemical splash goggles are also required. All operations involving dry asbestos fibers must be conducted in a certified ducted Biological Safety Cabinet or a properly operating and certified chemical fume hood. In our institution, mice are maintained in disposable cages in an isolated carcinogen room to facilitate the care of the animals while reducing the risk to the staff involved in the experiments. Alternatively, disposable cages may be used when mice are first injected with asbestos fibers and kept for 7 days in the carcinogen room in the chemical fume hood, after which they can be placed in regular cages and moved out of the carcinogen room to the main colony area. Syringes used for carcinogen injections must be safety-engineered (self-sheathing syringes, luer-lock syringes, etc.). Importantly, never transfer asbestos in powder form. Dry asbestos fibers can quickly become airborne, increasing the likelihood of inhalation.

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Preparation of Asbestos Solutions

1. Purchase UICC-grade asbestos from SPI Supplies (100 mg per container).
2. A fresh asbestos solution is prepared each time that mice are injected.
3. Unscrew the lid of the asbestos container, gently place a small bar magnet in the container, and screw back the lid, leaving it loosely covered so that the container will not crack while baking the asbestos. If the original asbestos container is too small to dissolve asbestos in the required volume, it should first be dissolved in about 5 ml of HBSS and then transferred to a larger sterile container to dilute further.
4. Place the whole container in a glass beaker, cover it with aluminum foil, affix indicator tape to the outside, and bake it in the oven.
5. Bake at 150°C for 18 h under 15 pounds of constant pressure to inactivate endotoxins that may be present in the asbestos.
6. The next day, let the oven cool to room temperature and check that the indicator sticker's color has changed. This ensures that the asbestos has baked appropriately.
7. Place the asbestos beaker under the chemical fume hood.
8. Unscrew the asbestos container, add the required amount of Hank's balanced salt solution (HBSS), and then mix for 30 minutes on a magnetic stirrer under the fume hood. Adding 62.5 ml of HBSS to 100 mg asbestos equals 1.6 mg/ml asbestos, and injecting 0.5 ml of this solution i.p. per mouse equals a dose of 0.8 mg.
9. After 30 minutes of mixing, use a sonicator or ultrasonic cleaner to dissolve the asbestos homogeneously for 40 minutes. *NOTE:* Wear earphone-type sound mufflers to protect your hearing while sonicating.
10. Place any contaminated paper towels, pipette tips, syringes, needles, gloves, asbestos containers, and vials in Ziploc bags. Double-bag them and deposit them in a red Sharps container marked "Carcinogens."

Asbestos Injections and Follow-up—*NOTE:* The endpoint of the experiment may vary depending on the investigator's goals. Our endpoint is typically tumor onset, evidence of illness, or weight loss greater than 10% of body weight (see further details below). In such cases, some animals may not succumb until more than 15 months, when the experiment will be terminated. Alternatively, the investigator may choose to terminate the experiment at a predetermined time point, e.g., 12 months, when most asbestos-exposed, untreated mice will have succumbed to mesothelioma.

1. Begin the study with 8−10-week-old *Bap1*$^{+/-}$ and WT littermates from the Basic Protocols detailed above to assess the tumorigenic effect of asbestos in the peritoneum. *NOTE:* Before the asbestos injections, mice are placed in disposable cages. Each group is assigned equal numbers of males and females.

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2. As mentioned, asbestos injection procedures are performed under a well-ventilated fume hood. The surface area is covered with plastic-backed absorbent paper towels to prevent asbestos from contaminating the hooded area.

3. Using a 1-ml syringe, collect 0.5 ml from the 1.6 mg/ml stock asbestos solution to inject each mouse.

4. Hold the mouse gently with your left hand so it is static, with its abdomen facing you. Swipe the abdominal area, focusing on the lower right quadrant, with 70% ethanol. Use your other hand to inject the 0.5 ml asbestos solution (0.8 mg asbestos) i.p. Note: Injecting the asbestos into the right lower quadrant of the abdomen minimizes the likelihood of puncturing any organs with the needle. For our standard asbestos dose, inject i.p. each mouse with 0.8 mg of freshly prepared crocidolite fibers in 0.5 ml HBSS using a 25-G needle. The injections are repeated every 21 days for a total of (0.8 mg/injection × 4 injections = 3.2 mg/mouse). The 21-day interval between injections was selected based on previous work showing that 20 μg to 1 mg of crocidolite injected i.p. stimulates mesothelial cell proliferation for at least 21 days (Macdonald & Kane, 1997). However, when using significantly lower doses (0.0125 to 0.1 mg/injection), we found that a few female heterozygous *Bap1*-mutant mice develop benign spontaneous sex cord-stromal tumors before succumbing to mesothelioma (Kadariya et al., 2016a). This could complicate the interpretation of the carcinogenicity results, although these are benign tumors that usually are not lethal.

5. Discard the used syringe in a red bin marked “Sharps, asbestos contaminated.” Gently wipe the mouse’s injected area with a new alcohol swab to remove asbestos particles around the injection site. Return the animal to a disposable cage.

6. Continue injecting all mice as in steps 1–5.

7. Collect all used swabs, paper towels, and contaminated gloves in a Ziploc bag, double-bag them, and dispose of them in a red bag in the procedure room. Proceed to the anteroom and place all PPE in a red bag. Pack trash from the procedure room and anteroom in a cardboard box marked “asbestos-contaminated” and seal it with clear tape for disposal.

8. Change the disposable cages after 1 week after injection. Repeat the asbestos injections every 21 days for a total of 4 injections/mouse, totaling 3.2 mg asbestos/mouse.

9. After the four injections are completed, the disposable cages are changed every other week. All disposable cages, including bedding, should be packed in a red bag like other contaminated objects.

10. Record the body weight of each animal weekly.

11. Examine all mice daily and sacrifice any mouse upon evidence of labored breathing, severe weight loss (>10% of body weight), abdominal bloating,

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lethargic behavior, hunched back, and/or difficulty in walking, or when tumor burden was evident, following a protocol approved by the IACUC at the investigator's institution. Mice are sacrificed by CO_2 asphyxiation followed by cervical dislocation.

12. At necropsy, histopathologically examine all organs for evidence of tumor lesions or overt malignancy. To evaluate tumor invasiveness and spreading, perform complete necropsies on all mice by examining the thoracic, abdominal, and pelvic cavities for tissue collection. Collect the tumor tissues in 10% buffered formaldehyde, fix them for 24–48 hours, and then process them for histopathologic examination by a qualified experimental pathologist. Collect a portion of each tumor and any ascitic fluids for storage at −80°C for further analysis, e.g., for molecular studies.

13. The presence of mesothelioma is scored based on histological and immunohistochemistry (IHC) evidence for staining with three or more mesothelioma markers (e.g., WT1, mesothelin, Troma 1). Formalin-fixed, paraffin-embedded (FFPE) sections are subjected to heat-induced epitope retrieval for IHC. Endogenous peroxidase activity is quenched with 3% hydrogen peroxide, and non-specific protein binding is blocked with goat serum before incubating the tumor sections with primary monoclonal antibodies.

    To evaluate differences in the incidence and median survival of mice with mesotheliomas among experimental groups, use Fisher's exact and log-rank tests, respectively. Kaplan–Meier curves are also prepared using GraphPad Prism software using data from Excel. An example of Kaplan–Meier survival curves in asbestos-treated *Bap1*$^{+/W}$, *Bap1*$^{+/L}$, and *Bap1+/+* (WT) mice is shown in Fig. 8.

BASIC PROTOCOL 4: PRECLINICAL CHEMOPREVENTION AND CHEMOTHERAPY STUDIES USING A GEMM WITH ASBESTOS-INDUCED MESOTHELIOMA

For these studies, Basic Protocol 1 would be used to generate the mice and expose cohorts to asbestos before intervention with preventive or therapeutic agents. The Materials are identical to the items mentioned above except for items listed in the Materials listed below. The timing and conditions vary depending on the goals of the investigation. In general, however, the timing of the intervention should depend on pilot studies aimed at identifying when tumor onset occurs in the model. For chemoprevention studies, the investigator would seek to begin treatment with a preventive agent soon after starting the first or second (of four) asbestos injections. For early interception, one might start treatment soon after the fourth injection or when pilot studies have shown areas of the peritoneal lining with thickening of the mesothelial lining. For therapeutic studies, treatment may begin when evidence of tumor invasion is evident, based on MRI studies, if available, or sacrifice of mice at different time points. The protocol below describes how a preclinical chemoprevention trial is performed on an asbestos-exposed GEMM with a germline heterozygous mutation of *Bap1*.

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Materials

The materials used to generate the mice are essentially the same as those in Basic Protocols 1 and 2, except for the following additional item, which is needed in Basic Protocol 3.

Reusable Animal Feeding Needles 22 gauge (Cadence Science, AFN 22g × 1”, 1.25 mm (straight), cat. no. SKU 7901)

1. Expand the *Bap1*$^{+/mut}$ colony that will be injected i.p. with asbestos and randomly assigned to treatment groups.
2. Meet with an experienced statistician to determine the number of *Bap1*$^{+/mut}$ mice required to perform a statistically meaningful three-arm study: vehicle-treated, lower-dose, and higher-dose treatment groups.
3. When mice are ~9 weeks old, asbestos injections are performed. The injections are given as described in Protocol 1 above (0.8 mg/injection × 4 injections every 21 days; total = 3.2 mg).
4. Begin drug (e.g., anakinra) treatments 2 days before the second asbestos injection and continue at intervals recommended in the literature for the drug of choice, potentially until the time of sacrifice when mice reach about 60–70 weeks of age.
5. Record each mouse’s weight before the first injection of crocidolite and every week after that. Keep mice in disposable cages in a procedure room designated for asbestos work if feasible. Change the cages one week after each asbestos injection. After completing the four injections, change the disposable cages every other week.
6. Two days before the second asbestos injection, randomize the mice into three treatment groups. The rationale for giving one dose of asbestos before starting treatment with the chemopreventive agent is that establishing chronic inflammation in the mouse peritoneal cavity would mimic the situation in humans, who will likely enter a clinical chemoprevention protocol after having some prior asbestos exposure. Control mice will receive vehicle (e.g., sterile H_2O). Drug treatments may be administered orally by gavage daily or several days per week (e.g., M, W, F) and continue until the time of sacrifice.
7. Examine the mice daily. Euthanize via CO_2 asphyxiation, followed by cervical dislocation, when palpable or visible masses arise or upon visible signs of distress, including extreme fatigue and labored breathing, or when mice exhibit a 15% change in body weight (humane endpoint). Euthanize the mice when their body weight increases by 15% (due to ascites or tumor) or decreases due to cachexia caused by tumor burden.
8. Upon sacrifice, the mice should be grossly examined for the presence of a solid tumor. Collect tumors, with one half used to prepare an FFPE sample for histopathological evaluation and the other half snap frozen for molecular biological analyses. All mice should also have all major internal organs collected, fixed in formalin, and subjected to histopathological assessments for

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evidence of mesothelioma dissemination. Other vital organs (liver, kidneys, pancreas, spleen, heart) should be examined grossly and histologically for potential damage indicative of drug toxicity as well as for tumor dissemination. The tumor tissue should be evaluated histopathologically to determine each treatment arm's incidence and histopathological type of mesothelioma. Survival of each mouse with mesothelioma is recorded for Kaplan-Meier curve analysis. All tumors should be processed for histological assessment and immunohistochemistry (IHC), including staining for WT1 and mesothelin to help verify mesothelioma diagnosis.

9. Fisher's exact and log-rank tests are used to assess the statistical significance of differences among mouse treatment groups regarding mesothelioma incidence and median survival, respectively.

BASIC PROTOCOL 5: GENERATION OF A GEMM WITH CONDITIONAL KNOCKOUT OF *Bap1*

This protocol describes how to generate a GEMM with floxed (f) alleles of *Bap1* via Cre-mediated somatic site-specific recombination to circumvent potential problems such as embryonic lethality due to germline homozygous deletion of the gene (Akagi et al., 1997). Using this Cre-LoxP system, the locotemporal knockout of a gene of interest is accomplished by injecting adenoviruses expressing Cre recombinase (Akagi et al., 1997). To generate a loss of a TSG such as *Bap1*, specifically in the mesothelial lining of the pleura or peritoneum, adenovirus expressing Cre recombinase (Ad5CMVCre, adeno-Cre) is injected into the pleural or peritoneal space, respectively, of mice harboring floxed *Bap1* alleles.

The protocol below describes how we generated a CKO of the *Bap1* gene using ZFN technology. As new TSGs are implicated in mesothelioma in the future, a similar approach could be used to design an appropriate knockout model for these other genes.

Materials

Custom ZFNs targeting the *Bap1* gene(Sigma-Aldrich)

Adenovirus (Ad5CMVCre)(Viral Vector Core, University of Iowa, Iowa City, IA, cat. no. VVC-U of Iowa-5)

FVB/N one-cell embryos

CD-1 mice

Tris-Borate-EDTA buffer, 10X (Santa Cruz Biotechnology, cat. no. sc-296650)

Agarose: PR1MA Agarose (Midsci, cat. no. MIDSCI-500)

SmartCheck DNA Ladders (MidSci, cat. no. PR4010)

Ethidium Bromide Solution 10 mg/mL (ThermoFisher Scientific, cat.no 17898)

Alcohol swabs (Fisher Scientific, cat. no. 1223K92)

Gentra Puregene Tissue Kit (Qiagen, cat. no. 158063)

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PfuTurbo DNA Polymerase (Agilent Technologies cat. no. 600250)

dNTP Mixture (Takara, cat. no. 4030)

Bap1 forward and reverse targeting primers (Integrated DNA Technologies IDT)

Thermal cycler

QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704)

DNA sequencing (Genewiz, Azenta Life Sciences)

Direct Mouse Genotyping kit (APExBIO, cat. no. K1025)

mBAP1-E6–1F (forward) and mBAP1-I7–4R (reverse) genotyping primers

GoTaq Green Master Mix (Promega, cat. no. M712)

Exel International Insulin Syringes (Fisher Scientific, cat. no. 14–841-31)

PPE (disposable gown, head cover, gloves, safety glasses, N95 mask, shoe covers)

3M N95 Healthcare Particulate Respirators and Surgical Masks, 1860 Series (Fisher Scientific, cat. no. 18–992)

MicroGuard MP, Microporous Coverall with attached hood & boot, elastic wrist, elastic back, open ankle (Thomas Scientific, cat . no. 21A00P353–8019)

Face Shield (Fisher Scientific, cat. no 19–181-800C)

Ansell MICROFLEX SafeGrip SG-375 (Fisher Scientific, cat. no. 19–048-575D)

Ketamine-acepromazine-xylazine cocktail

1. Design custom ZFNs targeting the *Bap1* gene. This step is identical to that described in Basic Protocol 1, step 1. Specifically, the custom ZFNs target a *Bap1* sequence with an intronic cutting site in intron 5 of *Bap1*. As in Basic Protocol 1, use Sigma-Aldrich's custom service to create the ZFN expression plasmids. The sequence for the binding and cutting sites of the ZFNs is TGCTCCCCAGTAGTCACTGATGGCTGTGCACG, with the spacer between the binding sites underlined. Sigma-Aldrich will validate the targets in mammalian cells.
2. Design a donor DNA construct containing LoxP sites in *Bap1* introns 6 and 7, such that adenovirus-mediated expression of Cre recombinase results in deletion of *Bap1* exon 7.
3. Combine ZFN mRNAs and donor DNA, inject into the pronucleus of one-cell embryos of FVB/N mice, and transfer those embryos into pseudopregnant females.
4. Clip the pups' tails from the pseudopregnant mice and isolate and purify DNA from the tail tips using a Puregene Tissue Kit (Qiagen).
5. Perform a PCR and gene sequencing analysis to verify correct gene targeting. For PCR, use forward primer *Bap1*$^{f/f}$ 1062: 5′-

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AGGCTTTGCTGCTAAATGAGA-3′ and reverse primer *Bap1*$^{f/f}$ 1063: 5′-CCCTGAGACCCAGAAAATCA-3′.

10X PfuTurbo buffer	2.5 µl
dNTPs	2.5 µl
Forward primer 1062 10 µm	1.0 µl
Reverse primer 1063 10 µm	1.0 µl
PfuTurbo DNA polymerase	0.5 µl
Nuclease Free Water	16.5 µl
DNA (100 ηg/µl)	1.0 µl
Total Volume	25.0 µl

6. Perform PCR as follows: 95°C (5 minutes), followed by 30 cycles at 95°C for denaturation (30 seconds), 60°C for annealing (30 seconds), and 72°C for extension (45 seconds), with a final extension at 72°C (10 minutes).

7. Resolve PCR products on a 1% agarose gel for 1 h and extract DNA from the gel using a QIAquick Gel Extraction Kit (Qiagen). The product size of the WT allele is 634 bp, and the size for the floxed allele is 702 bp. (Fig. 9A). The 702 bp fragment is gel-purified for Sanger sequencing to identify founder mice with integrated LoxP sites.

8. After sequence analysis, founder mice with LoxP sites were identified and integrated into the *Bap1* locus, one of which is described here.

9. After identifying the founder line, genotype the offspring by extracting DNA from mouse tails using a Direct Mouse Genotyping Kit (APExBIO). To genotype *Bap1*$^{f/f}$ mice, we now use a new set of primers for PCR that results in a smaller product better suited for differentiating between the WT and floxed alleles: mBAP1-E6–1F (forward): 5’-GAACTGGCCAAGGCACATAA-3’ and mBAP1-I7–4R (reverse): 5’-ACCCAGAAAATCAGAAGGAAGCAT-3’.

2X GoTaq Green Master Mix	10 µl
mBap1-E6–1F primer 10 µm	1 µl
mBap1-I7–4R primer 10µm	1 µl
Nuclease free water	7 µl
DNA	1 µl
Total Volume	20 µl

Thermal PCR cycling conditions with the new primers are 95°C (5 minutes), followed by 29 cycles of 95°C for denaturation (30 seconds), 60°C for annealing (30 seconds), and 72°C for extension (30 seconds) with a final extension at 72°C (5 minutes). The product size of the wt allele is 307 bp, and the size for the floxed allele is 375 bp. (Fig. 9B).

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BASIC PROTOCOL 6: GENERATION OF A CONDITIONAL KNOCKOUT MODEL OF MESOTHELIOMA

This protocol describes how a GEMM with floxed (f) alleles of two or more TSGs, e.g., *Bap1*, *Nf2*, and *Cdkn2a*, implicated in human mesothelioma pathogenesis is generated to induce a high incidence of mouse mesotheliomas in the absence of carcinogenic exposure to asbestos (Jongsma et al., 2008; Sementino et al., 2018; Kukuyan et al., 2019; Badhai et al., 2020). Using the Cre-LoxP system, the locotemporal knockout of multiple TSGs of interest is accomplished by injecting adenovirus expressing Cre recombinase (adeno-Cre) into a target tissue site (Akagi et al., 1997). To induce mesothelioma, adeno-Cre is injected into the pleural or peritoneal space of mice with floxed alleles. In their seminal report, Berns and colleagues demonstrated a high frequency of pleural mesotheliomas after injecting adeno-Cre into the pleural space of conditional knockout (CKO) mice with either homozygously floxed *Nf2* and *Tp53* or *Nf2* and *Cdkn2a*, with median survival times of about 30 and 20 weeks, respectively (Jongsma et al., 2008). These and subsequent GEMMs with the additional knockout of *Bap1* (Kukuyan et al., 2019; Badhai et al., 2020), now known to be the most frequently mutated gene in human mesothelioma, closely mimic the phenotype of human pleural mesothelioma. Moreover, injecting adeno-Cre into the pleural space of $Bap1^{ff};Nf2^{ff};Cdkn2a^{ff}$ mice (Kukuyan et al., 2019) or $Bap1^{ff};Nf2^{ff};Cdkn2ab^{-/-}$ mice (Badhai et al., 2020) each exhibited a high penetrance (≥ 85%) of mesotheliomas with median survival times of about 12 weeks. Thus, such CKO models provide a rapid model system for preclinical testing of novel targeted therapies and new drug combinations, including immunotherapies, without the need for asbestos.

In this protocol, $Bap1^{f/f}$ mice (Basic Protocol 5) are crossed to $Nf2^{f/f};Cdkn2a^{f/f}$ mice for studies of tumorigenic cooperativity of critical driver genes in mesothelioma pathogenesis. $Nf2^{f/f};Cdkn2a^{f/f}$ mice (Jongsma et al., 2008) were a gift from Anton Berns (Netherlands Cancer Institute, Amsterdam, The Netherlands). Although Dr. Berns has retired, the mice are available through a material transfer agreement (MTA) from the Netherlands Cancer Institute's Office of Knowledge Transfer & Contracting (Frank Hoorn, Office Manager, f.hoorn@nki.nl). Floxed *Cdkn2a* mice (B6.129P2-Cdkn2^{atm2Brn}/A), developed in Anton Berns' laboratory, are also available commercially from Taconic Biosciences. Our mice were maintained in a mixed FVB/N × 129/Sv background. The LoxP sites in the *Cdkn2a* locus of these mice permit the excision of exon 2, which results in the inactivation of both of its gene products, p16Ink4a and p19Arf. $Nf2^{f/f};Cdkna^{f/f}$ and $Bap1^{f/f}$ mice were crossed to generate cohorts with the following genotypes: $Bap1^{f/f}$, $Nf2^{f/f}$, $Cdkn2a^{f/f}$, $Bap1^{f/f};Nf2^{f/f}$, $Bap1^{f/f};Cdkn2a^{f/f}$, $Nf2^{f/f};Cdkn2a^{f/f}$, and $Bap1^{f/f};Nf2^{f/f};Cdkn2a^{f/f}$.

Materials

$Bap1^{f/f}$ mice

$Nf2^{f/f}$ mice

$Cdkn2a^{f/f}$ mice

mBAP1-E6–1F (forward) and mBAP1-I7–4R (reverse) genotyping primers (Integrated DNA Technologies, IDT)

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Genotyping primers for $Nf2^{f/f}$ mice (Integrated DNA Technologies)

Genotyping primers for *Cdkn2a*$^{f/f}$ mice (Integrated DNA Technologies)

Thermal cycler

GoTaq Green Master Mix

Adenovirus (Ad5CMVCre) (Viral Vector Core, University of Iowa, cat. no. VVC-U of Iowa-5)

Econo-Cage Disposable System (irradiated) with air grommet (Lab Products, cat. no. LPI no. 72014-GI)

Tris-Borate-EDTA buffer, 10X (Santa Cruz Biotechnology, cat.no. sc-296650)

Agarose: PR1MA Agarose (Midsci, cat. no. MIDSCI-500)

SmartCheck DNA Ladders (MidSci, cat. no. PR4010)

Ethidium Bromide Solution 10 mg/mL (ThermoFisher Scientific, cat. no. 17898)

Alcohol swabs (ThermoScientific, cat. no. 1223K92)

Direct Mouse Genotyping kit (APExBIO, cat. no. K1025)

GoTaq Green Master Mix (Promega, cat. no. M712)

Exel International Insulin Syringes (Fisher Scientific, cat. no 14–841-31)

PPE (disposable gown, head cover, gloves, safety glasses, N95 mask, shoe covers)

3M N95 Healthcare Particulate Respirators and Surgical Masks, 1860 Series (Fisher Scientific, cat. no. 18–992)

MicroGuard MP, Microporous Coverall with attached hood & boot, elastic wrist, elastic back, open ankle (Thomas Scientific, cat. no. 21A00P353–8019)

Face Shield (Fisher Scientific, cat. no. 19–181-800C)

Ansell MICROFLEX SafeGrip SG-375 (Fisher Scientific, cat. no. 19–048-575D)

Ketamine-acepromazine-xylazine cocktail

10% buffered formaldehyde

Tissue-Plus O.C.T. Compound (Fisher Healthcare, cat. no. 23–730-571)

RNA*later* Solution (Thermo Fisher Scientific)

Anti-Mesothelin Polyclonal Antibody (Thermo Fisher Scientific, cat. no PA5–79698),

Anti-Wilms Tumor Protein (WT1) antibody [CAN-R9(IHC)-56–2] (Abcam, cat. no. ab89901)

Anti-Cytokeratin 8 antibody, clone TROMA-1 (Sigma-Aldrich MABT329M)

GraphPad Prism Software (Dotmatics)

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Genotyping of mice with floxed alleles of *Nf2 and Cdkn2a*

1. *Nf2*$^{f/f}$1048 (forward): 5′-CTT CCC AGA CAA GCA GGG TTC-3′ and *Nf2*$^{f/f}$ 1049 (reverse): 5′-GAA GGC AGC TTC TTC CTT AAG TC-3′ with the following PCR reagents.

2X GoTaq Green Master Mix	10 µl
Nf2$^{f/f}$ 1048 primer 10 µm	1 µl
Nf2$^{f/f}$ 1049 primer 10µm	1 µl
Nuclease free water	7 µl
DNA	1 µl
Total Volume	20 µl

Thermal PCR cycling conditions used are 95°C (5 minutes), followed by 29 cycles of 95°C for denaturation (30 seconds), 60°C for annealing (30 seconds), and 72°C for extension (30 seconds), with final extension at 72°C (5 minutes). The product size for the wild-type allele is 305 bp, and the size for the floxed allele is 442 bp. The PCR reaction is as follows, and a representative example of the genotyping is shown in Fig. 10A.

2. *Cdkn2a*$^{f/f}$ 1025 (forward): 5′-GCA GTG TTG CAG TTT GAA CCC-3′ and *Cdkn2a*$^{f/f}$ 1026 (reverse): 5′-TGT GGC AAC TGA TTC AGT TGG-3′ with the following reagents.

2X GoTaq Green Master Mix	10 µl
Cdkn2a$^{f/f}$ 1025 primer 10 µm	1 µl
Cdkn2a$^{f/f}$ 1026 primer 10µm	1 µl
Nuclease free water	7 µl
DNA	1 µl
Total Volume	20 µl

Thermal PCR cycling conditions used are 95°C (5 minutes), followed by 29 cycles at 95°C for denaturation (30 seconds), 60°C for annealing (30 seconds), and 72°C for extension (40 seconds) with a final extension at 72°C (5 minutes). The product size for the wild-type allele is 490 bp, and the size for the floxed allele is 600 bp. The PCR reaction is as follows, and a representative example of the genotyping is shown in Fig. 10B.

3. Genotyping of mice with floxed alleles of *Bap1* is performed as in Basic Protocol 5, Step 9, using primers mBap1-E6–1F and mBap1-I7–4R.

Intrapleural injections of adeno-Cre and follow-up of mice—To excise floxed alleles of *Bap1*, *Nf2*, and *Cdkn2a*, specifically in the mesothelial lining of the pleural cavity, inject adeno-Cre (Ad5CMVCre, VVC-U of Iowa-5) virus into the pleural cavity. The

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adenovirus can be obtained from the Viral Vector Core of the University of Iowa (Iowa City, IA). This adenovirus expresses Cre recombinase, which excises targeted alleles by cutting at the LoxP sites surrounding the specific gene(s) of interest. Depending on the study, cohorts of mice with different combinations of floxed alleles may be used. In the protocol below, *Bap1*$^{f/f}$;*Nf2*$^{f/f}$;*Cdkn2a*$^{f/f}$ mice are used.

1. Discuss the study details with a biostatistician to determine the specific controls and number of mice per experimental arm needed to address the study goals.
2. Breeding is carried out so that equivalent numbers of male and female *Bap1*$^{f/f}$;*Nf2*$^{f/f}$;*Cdkn2a*$^{f/f}$ mice, 8–10 weeks of age, will be available to begin the experiment, which may occur batched, if necessary.
3. Draw a 50 μL solution containing 3–6 × 10^{10} PFU viral particles into a 1 ml insulin injection syringe with a needle, then place on an alcohol swab.
4. Sedate the mouse temporarily using an i.p. injection of ketamine: xylazine mixture (200 μL per 20 g mouse).
5. When sedated, hold the mouse in the left hand, supporting its back with the thumb and placing the forefinger behind the head to fix the chest.
6. Clean the injection site (right chest) with an alcohol swab (70% ethanol), and carefully insert the needle between the ribs and no more than 2–3 mm inside the chest cavity, followed by injection of the 50 μL viral particle suspension.
7. After the injection, place the animal in a regular cage and monitor it until it is fully recovered from the sedative agents. On the cage, place a biohazard card marked “Adeno-Cre injected.”
8. Repeat steps 3–7 for the remaining mice.
9. Dispose of all used syringes and needles in a red bin with a cover.
10. After a week, change the cages and bedding. The used supplies, including any disposable cages and bedding, should be disposed of in a red biohazard bag packed in a cardboard box for incineration.
11. Monitor all mice daily and euthanize animals upon signs of distress, including extreme fatigue, labored breathing, abdominal bloating, hunched back, difficulty walking, or when mice exhibit a 10% change in body weight. Collect tissues of all organs of the pleural and peritoneal cavities from sacrificed mice in 10% buffered formaldehyde, fix for 24–48 hours, and subject tumor specimens to histopathologic and IHC analysis. Place portions of tumors in an embedding medium (Fisher Healthcare Tissue-Plus O.C.T. Compound and RNA*later* Solution and immediately freeze at −80°C.
12. The histopathologic procedures used are the same as described above in Basic Protocol 3, Step 13. In brief, mesothelioma diagnosis is based on histological and IHC evidence for staining with mesothelioma markers, WT1, mesothelin, and Troma 1. FFPE tumor sections are subjected to heat-induced epitope retrieval for IHC. Endogenous peroxidase activity is quenched with 3% hydrogen peroxide,

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and non-specific protein binding is blocked with goat serum before incubating the tumor sections with primary monoclonal antibodies.

13. To evaluate differences in the incidence and median survival of mice with mesotheliomas among experimental groups, use Fisher's exact and log-rank tests, respectively. Kaplan–Meier curves are also prepared.

CAUTION: There is a mortality risk due to the needle's accidental rupture of the visceral layer of the pleura. However, the risk is very low if the injection procedure is carefully performed.

UNDERSTANDING OF RESULTS:

As shown in the Kaplan–Meier survival curves presented in Fig. 8, asbestos-treated *Bap1*-mutant mice succumb to disease earlier than asbestos-treated WT littermates, with a median survival of 46 and 48 weeks from the time of the first asbestos injection in $Bap1^{+/L}$ and $Bap1^{+/W}$ mice, respectively, compared to 60 weeks in WT mice ($p < 0.01$). The deaths due to peritoneal mesothelioma were 74% in $Bap1^{+/W}$ mice and 71% in $Bap1^{+/L}$ mice compared to 35% of WT animals, which was highly significant ($p < 0.01$).

As noted above, when asbestos experiments are performed on mice harboring compound heterozygous mutations or deletions, the timing may be relatively short because the mice develop rapid, aggressive tumors. Such compound heterozygous mice are better suited for preclinical chemotherapeutic, or potentially cancer interception, studies than long-term chemoprevention studies. In a chemoprevention experiment, $Nf2^{+/-};Cdkn2a^{+/-}$ mice exposed to asbestos in the presence of anakinra, an IL1 receptor antagonist, showed a marked delay in the median time of mesothelioma onset compared with similarly exposed mice given vehicle control (33.1 weeks vs. 22.6 weeks, respectively) (Fig. 11) (Kadariya et al., 2016b). However, despite the delayed onset of mesothelioma in anakinra-treated mice, all of the animals in this accelerated model of mesothelioma also died of the disease. Thus, a less aggressive model for chemoprevention experiments, e.g., asbestos-exposed heterozygous *Bap1*-mutant mice, would be preferable, as the test agent might be more likely to prevent some mesotheliomas from forming.

COMMENTARY

Critical Parameters:

In Basic Protocols 1, 2 and 5, the assistance of a dedicated institutional transgenic mouse facility is essential for fertilized egg isolations, pronuclear injections, and implantation of microinjected embryos. As noted above, specific factors can influence the protocol, and special attention to details should be paid to obtain robust results. First, staff performing asbestos work (Basic Protocols 3 and 4) must be adequately trained, and workers must be precise because asbestos is carcinogenic. SOPs and an isolated carcinogen room are essential to protect the staff and others who might use the same room. Training is necessary to minimize the chance that the injected asbestos enters the peritoneal space, not a vital organ.

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In Basic Protocols 5 and 6, experiments performed with CKO mice must also be carried out by well-trained staff according to SOPs. Sufficient hands-on training is required to minimize the chance that the needle used to inject adeno-Cre does not cause pneumothorax via rupture of the visceral layer of the pleura.

Time Considerations:

Asbestos carcinogenicity experiments can be lengthy if the GEMM has a heterozygous lesion in only a single TSG. The experiment can take a full year in many cases, but it is the primary way to demonstrate that a single gene can influence susceptibility to mesothelioma. Such a model may also be invaluable for chemoprevention studies, as a germline mutation in a single TSG, such as *Bap1*, mimics the situation in humans with a hereditary tumor susceptibility syndrome. The timing may be short when asbestos experiments are performed on mice with compound heterozygous mutations or deletions. For example, asbestos-induced mesotheliomas in $Nf2^{+/-};Cdkn2a^{+/-}$mice have a median survival of about 22 weeks (Menges et al., 2014).

Time considerations for experiments with CKO models of mesothelioma can vary depending on the number of excised genes and alleles, but they may generally be completed in 3–6 months.

ACKNOWLEDGMENTS

This work was supported by NCI grants CA175691, R03 CA280410, and CA06927, NCI contracts 6U24OH009077 and 75N91019D00022/Task Order 75N91023F00003, and by the Local #14 Mesothelioma Fund of the International Association of Heat and Frost Insulators and Allied Workers. The authors also wish to acknowledge the following Fox Chase Cancer Center shared resources that have assisted our work on mesothelioma mouse models over the years: Laboratory Animal, Transgenic Mouse, Histopathology, and Biostatistics and Bioinformatics Facilities.

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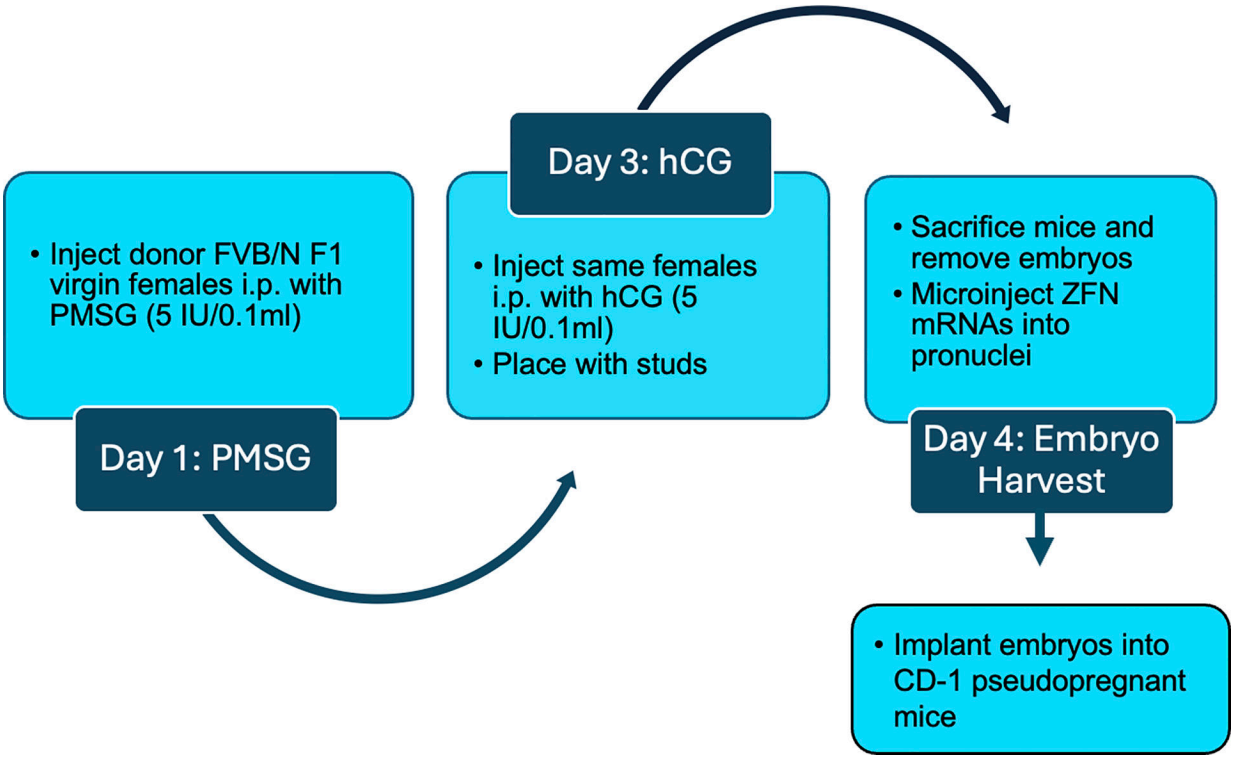

Figure 1.
Flow diagram of the procedures involved in the fertilized egg isolation, pronuclear injection, and implantation of microinjected embryos portion of Basic Protocol 1.

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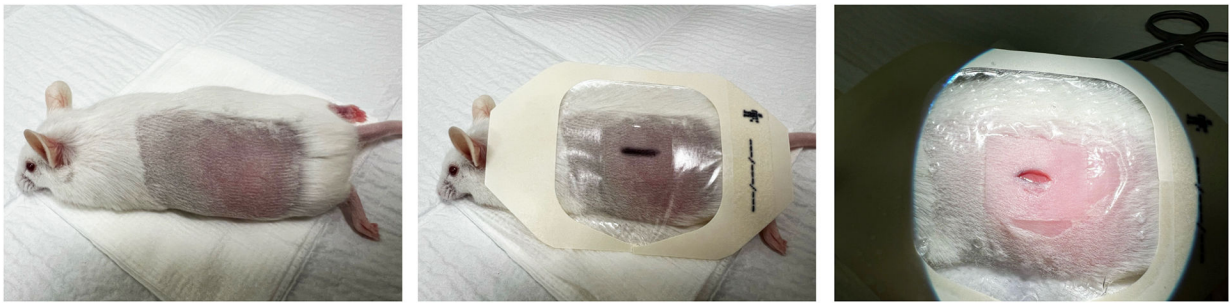

Figure 2.

Sequential images of an anesthetized, shaved pseudopregnant mouse (*left*) in which the surgical site has been disinfected and covered by 3M Tegaderm protective film and incision site marked (*middle*), and incision is then made (*right*).

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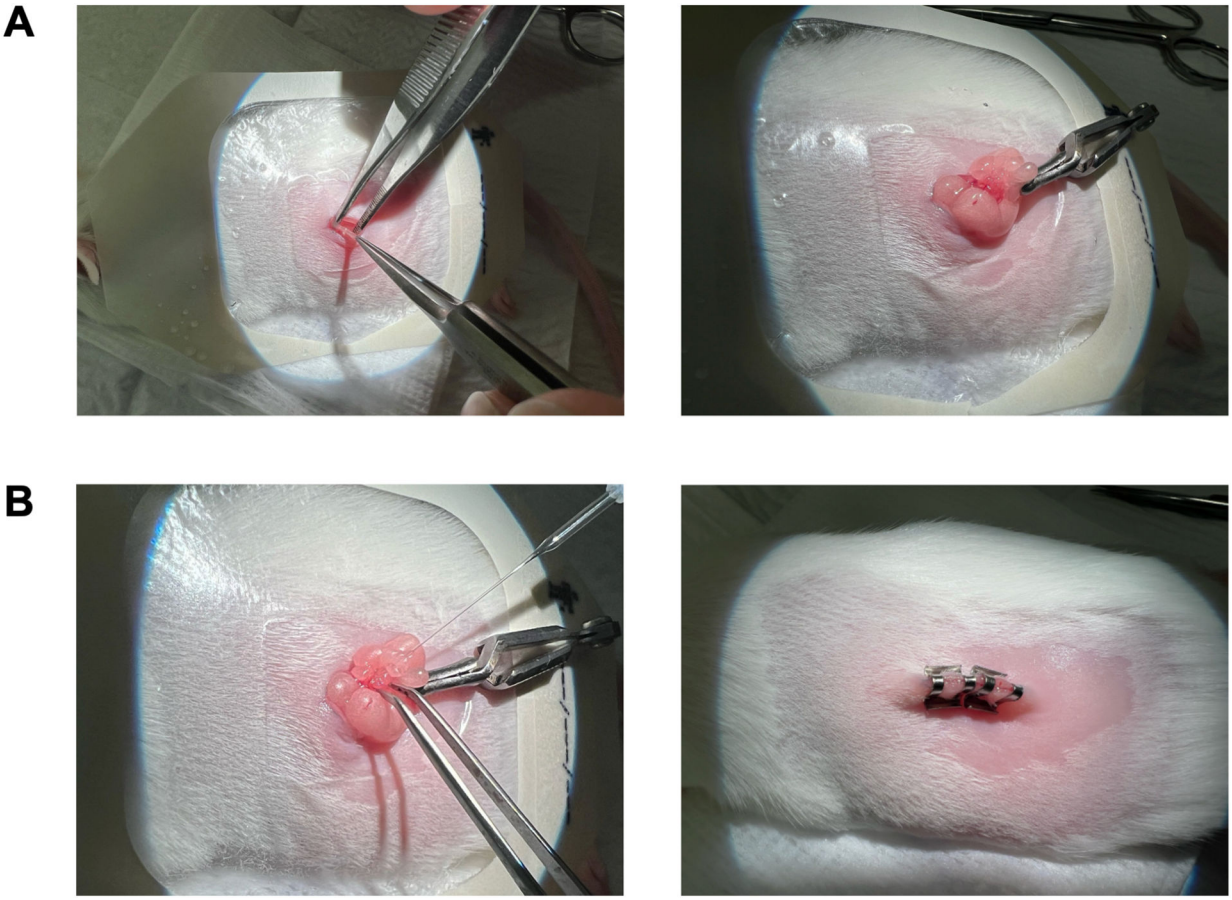

Figure 3.

After making the incision in the recipient pseudopregnant mouse (Fig. 1), the mouse body wall muscle is cut, and the ovary and oviduct complexes are exposed using forceps (**A**). The embryos are then implanted into the oviduct using a glass capillary tube, and the skin wound is closed by sutures and surgical clips **(B)**.

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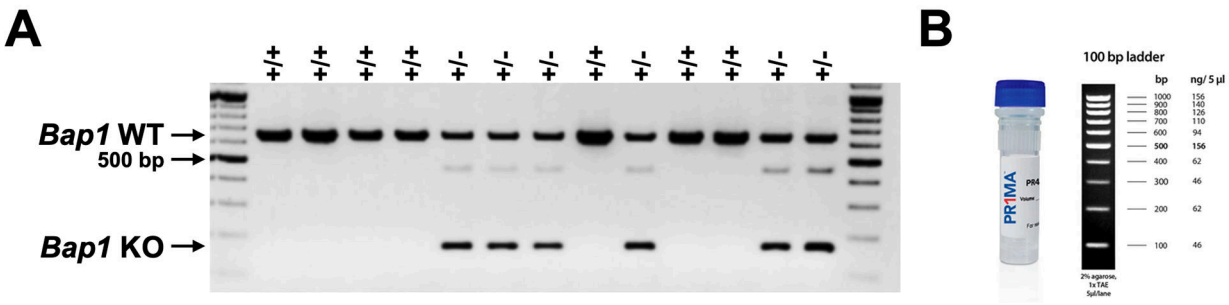

Figure 4.

A) Representative genotyping of tail DNA from mice with a germline heterozygous *Bap1* knockout (KO) allele (+/−) and littermates that are wild-type (WT, +/+) for *Bap1*. The size of the WT allele is 634 bp, whereas the size of the KO allele is 158 bp. **B)** A 100-bp DNA size marker ladder (PR1MA SmartCheck) was used for PCR analysis in panel **A**.

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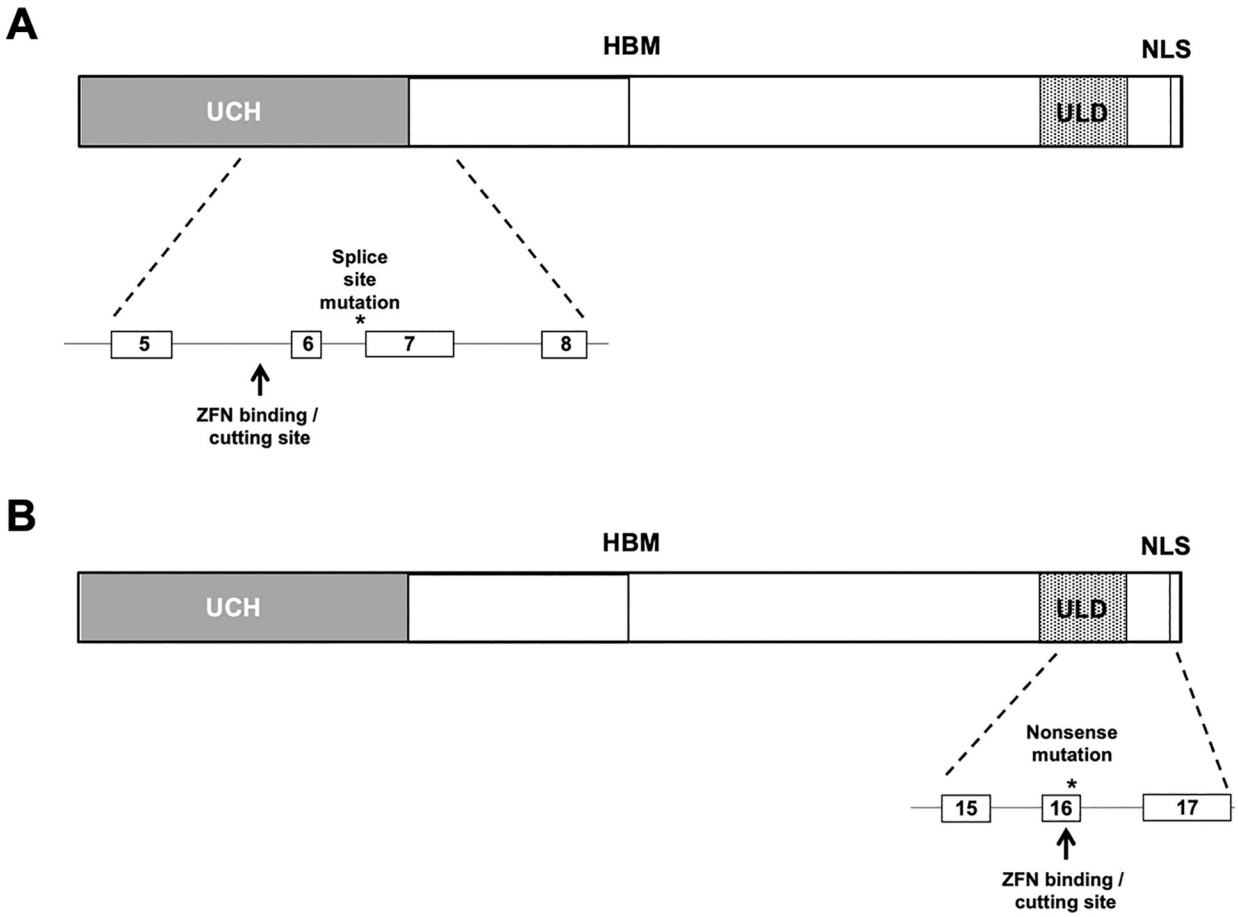

Figure 5.

Schematic diagrams showing cutting sites of the ZFNs and for *Bap1*$^{+/W}$ **(A)** and *Bap1*$^{+/L}$ **(B)** mutant mice. Asterisks (*) indicate splice site mutation in intron 6 of *Bap1*$^{+/W}$ and nonsense mutation in exon 16 of *Bap1*$^{+/L}$ mice, respectively. Modified from Kadariya et al., 2016a.

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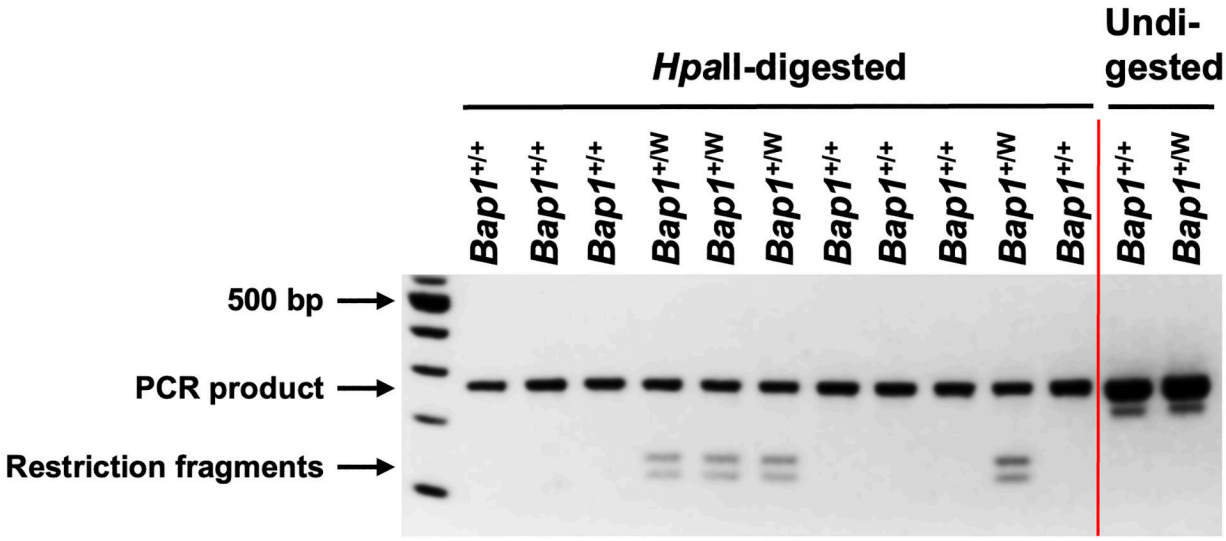

Figure 6.

Genotyping of heterozygous *Bap1*$^{+/W}$ knock-in mice. Genotyping of WT (+/+) and heterozygous (+/W) *Bap1* mice, the latter harboring a splice site mutation in intron 6. The PCR product size of both WT and mutant (knock-in) *Bap1* is ~290 bp. To detect the allele with the knock-in mutation, the PCR products are digested with *Hpa*II restriction enzyme, which results in two additional smaller restriction fragment bands in heterozygous (+/W) *Bap1* mice.

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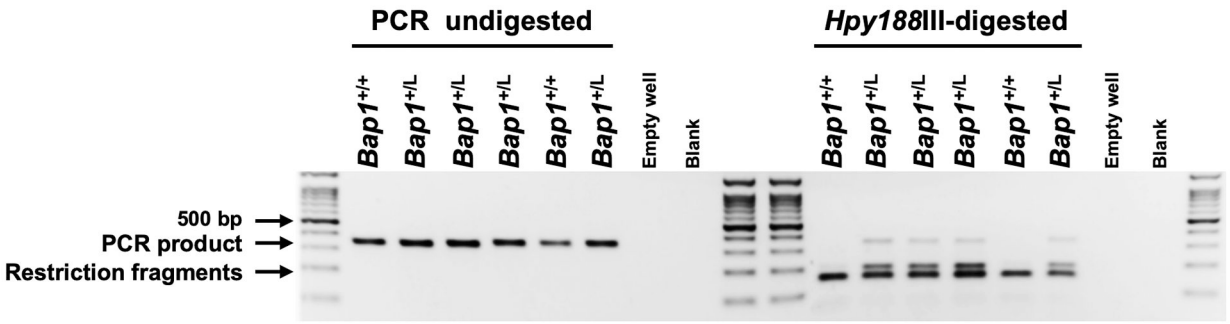

Figure 7.

Genotyping of the heterozygous *Bap1*$^{+/L}$ knock-in mice. Genotyping of WT (+/+) and heterozygous (+/L) *Bap1* mice, the latter harboring a nonsense mutation in exon 16. PCR products of tail DNA from both WT and *Bap1*$^{+/L}$ mice is 351 bp. To detect the *Bap1*-mutant allele with the knock-in mutation, the PCR products are digested with *Hpy188*III restriction enzyme, which produces two restriction fragment bands in the heterozygous (+/L) *Bap1* mice and only one fragment band in the WT mice.

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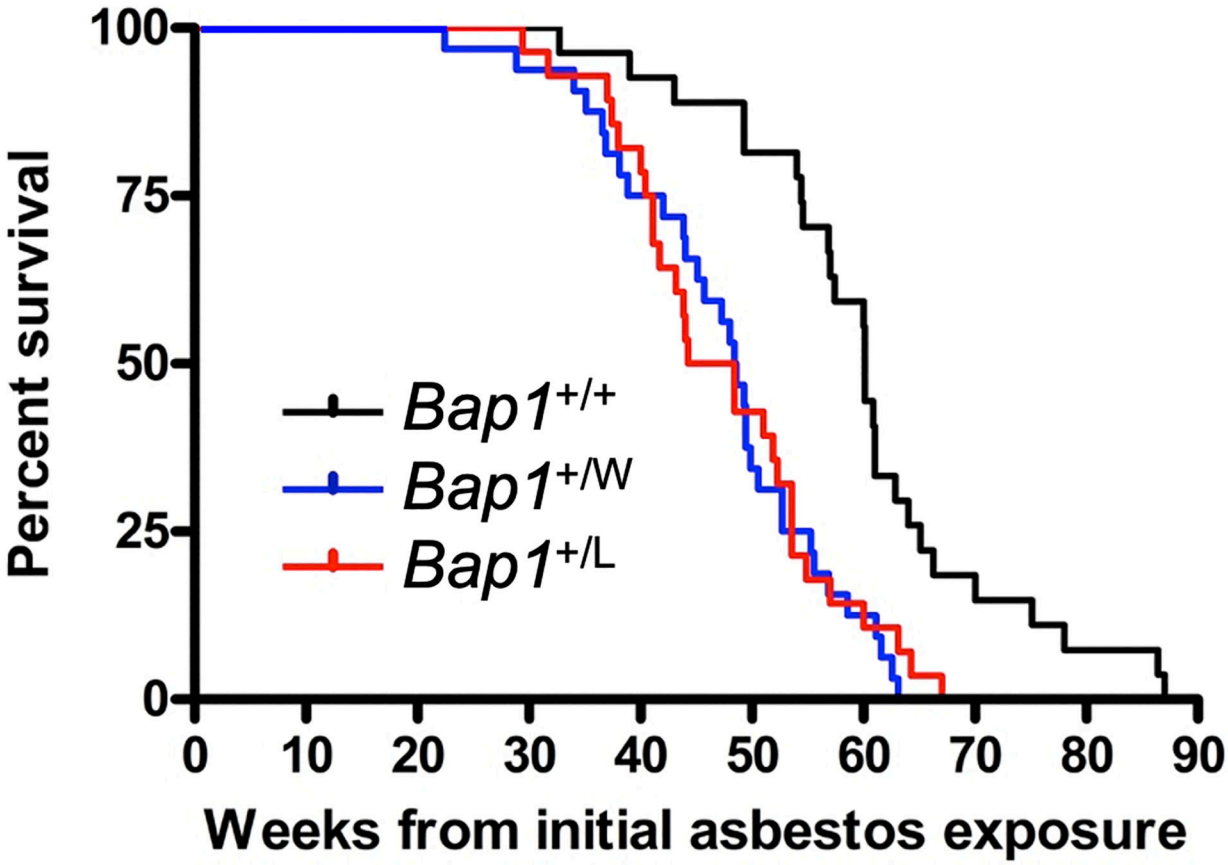

Figure 8.

Kaplan-Meier survival curves demonstrating markedly decreased survival of asbestos-exposed *Bap1*-mutant knock-in cohorts than in asbestos-exposed WT littermates. Survival differences were highly significant ($p < 0.01$ for WT mice vs. each $Bap1^{+/W}$ mice; $p < 0.008$ for WT vs. *Bap1*-mutant knock-in cohort). The percentage of deaths due to peritoneal mesothelioma was 74% in $Bap1^{+/W}$ mice and 71% in $Bap1^{+/L}$ mice compared to 35% of WT animals, which was highly significant ($p < 0.01$). From Kadariya et al., 2016a.

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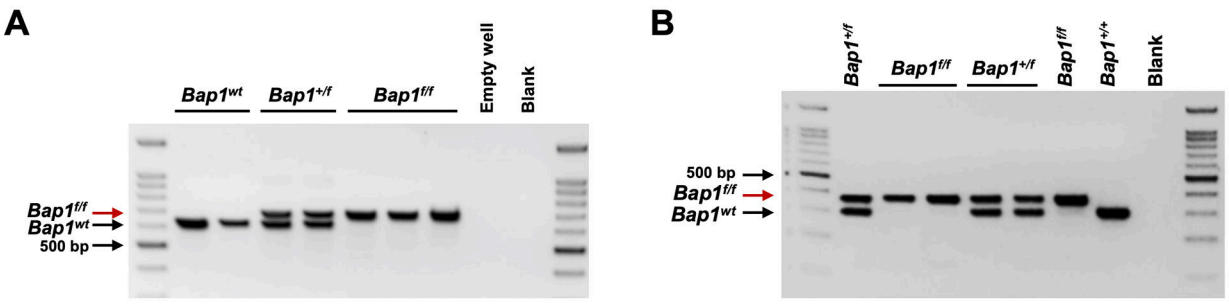

Figure 9.

Genotyping of mice with floxed allele(s) of *Bap1*. **(A)** PCR analysis with *Bap1* primers 1062 (forward) and 1063 reverse yielded products of the following sizes: *Bap1* wt allele (634 bp) and *Bap1* floxed (f) allele (red arrow, 702 bp). These primer pairs amplify both LoxP sites flanking exon 7 of the mouse *Bap1* gene (LoxP sites were inserted into introns 6 and 7.) Genotyping of presentative wild type (wt, +/+), heterozygous (+/f), and homozygous (f/f) conditional KO mice are shown. **(B)** Genotyping of mice with floxed allele(s) of *Bap1* using a new set of primers for PCR that provide smaller products better suited for differentiating between the wt and floxed allele. Representative genotyping of $Bap1^{+/+}$, $Bap1^{+/f}$, and $Bap1^{f/f}$ mice are shown. The size of the *Bap1* wt allele is 307 bp, whereas the size of the *Bap1* floxed (f) allele (red arrow) is 375 bp.

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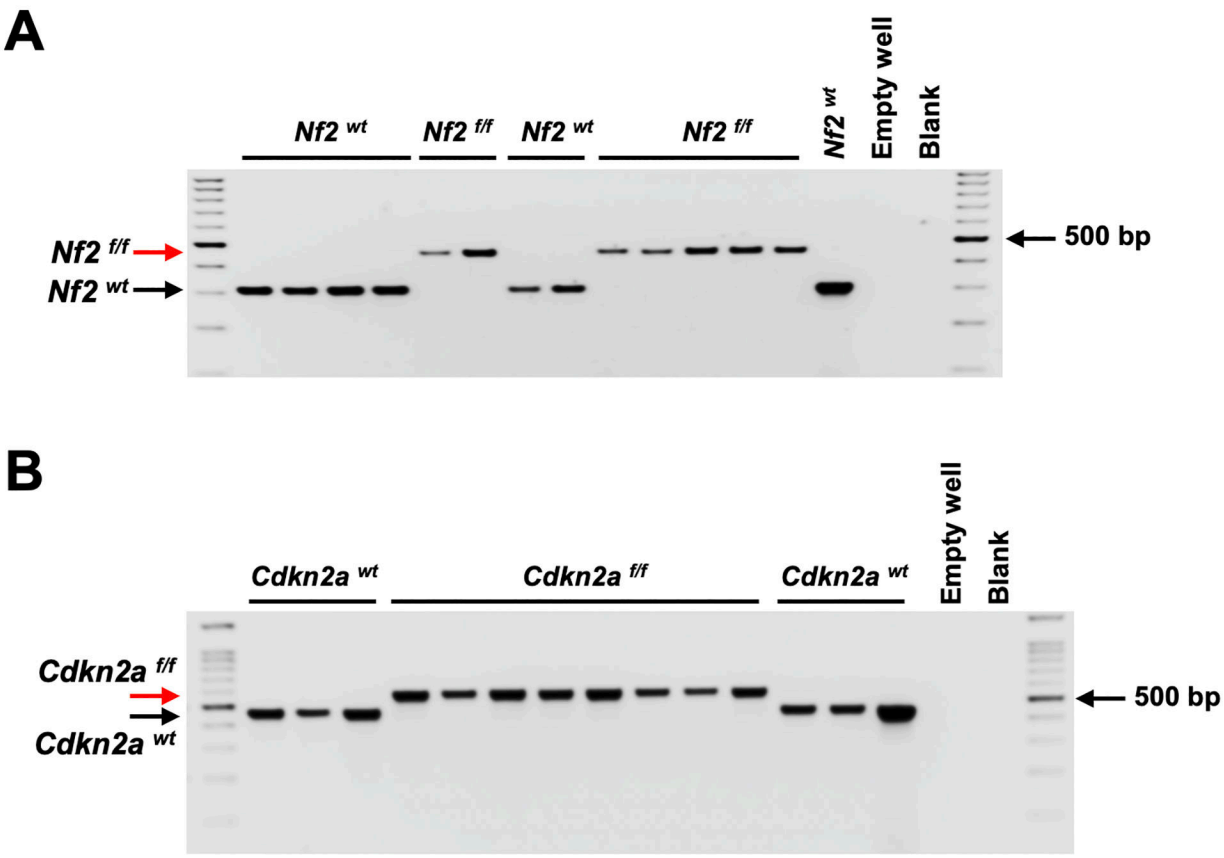

Figure 10.

Genotyping of mice with floxed alleles of *Nf2* and *Cdkn2a*. **(A)** Representative genotyping of homozygously floxed (f/f) *Nf2* conditional KO mice and wild-type (wt) littermates. The size of the *Nf2* wt allele is 305 bp, and the size of the floxed Nf2 allele (red arrow) is 442 bp. **(B)** Representative genotyping of homozygous *Cdkn2a* conditional KO mice and wt mice. The size of the *Cdkn2a* wt allele is 490 bp, whereas the floxed *Cdkn2a* floxed allele (red arrow) is ~590 bp.

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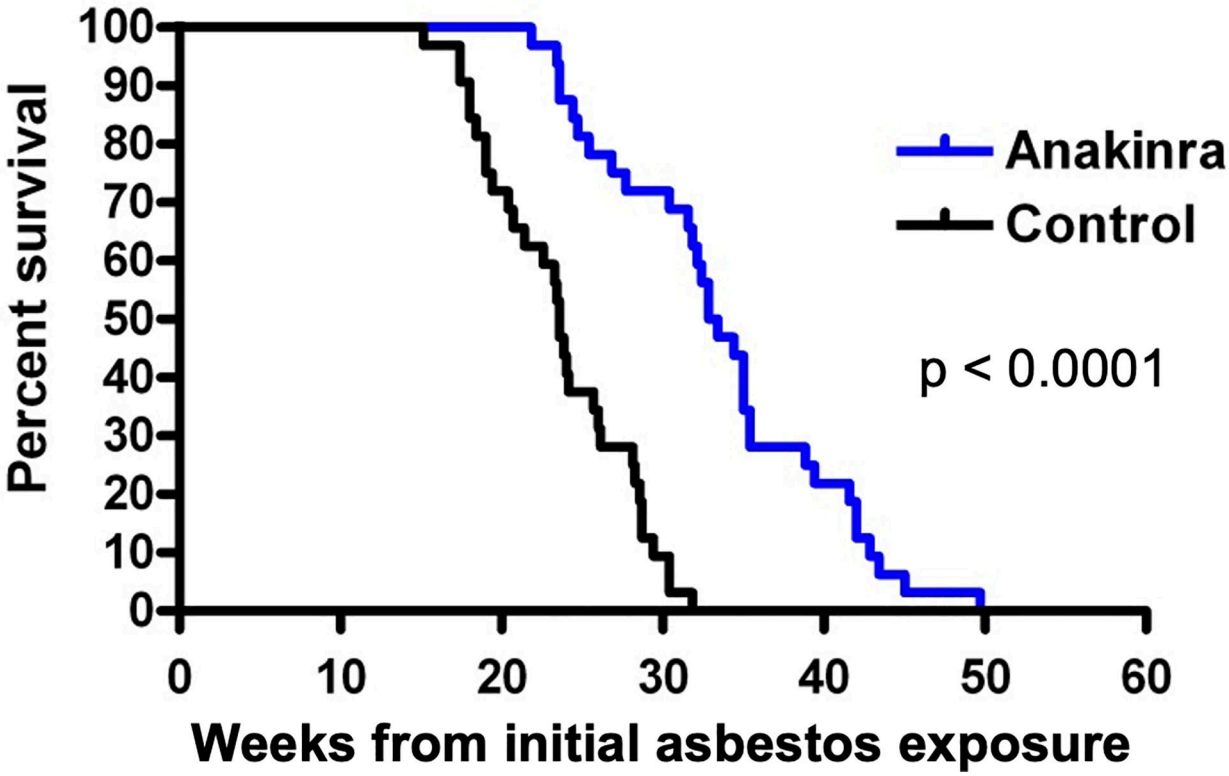

Figure 11.

To investigate the efficacy of anakinra as a chemopreventive agent, *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice were injected i.p. with 800 μg of crocidolite every 21 days × 4 injections, as in Basic Protocol 4. Anakinra (*Kineret*, Amgen), a human recombinant IL-1R antagonist, was dissolved in citrate buffer and diluted to 100 μg/100 μl in citrate buffer. Anakinra was injected i.p. at a concentration of 5 mg/kg body weight 6 h before the first asbestos injection. After each asbestos injection, anakinra was given every third day at the same concentration. In parallel, control *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice were injected i.p. with 100 μl of citrate buffer. A small gauge needle (21G for asbestos; 26G for anakinra or citrate buffer) and different injection sites were used so that one mouse area did not become overly sensitized. In this accelerated mouse model of mesothelioma, mice were followed for up to 50 weeks. Thirty-two *Nf2*$^{+/-}$;*Cdkn2a*$^{+/-}$ mice per arm were exposed to asbestos in the presence or absence of the IL-1R antagonist. The figure is modified from Kadariya et al., 2016b.