Establishment and analysis of a novel mouse line carrying a conditional knockin allele of a cancer-specific FBXW7 mutation

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F-box and WD40 domain protein 7 (FBXW7) is a component of the SKP1-CUL1-F-box protein (SCF) complex that mediates the ubiquitination of diverse oncogenic target proteins. The exploration of FBXW7 mutations in human primary cancer has revealed three mutation hotspots at conserved arginine residues (Arg465, Arg479, and Arg505) in the WD40 domain, which are critical for substrate recognition. To study the function of human FBXW7R465C, the most frequent mutation in human malignancies, we generated a novel conditional knockin mouse line of murine Fbxw7R468C corresponding to human FBXW7R465C. Systemic heterozygous knockin of the Fbxw7R468C mutation resulted in perinatal lethality due to defects in lung development, and occasionally caused an eyes-open at birth phenotype and cleft palate. Furthermore, mice carrying liver-specific heterozygous and homozygous Fbxw7R468C alleles cooperated with an oncogenic Kras mutation to exhibit bile duct hyperplasia within 8 months of birth and cholangiocarcinoma-like lesions within 8 weeks of birth, respectively. In addition, the substrates affected by the mutant Fbxw7 differed between the embryos, embryonic fibroblasts, and adult liver. This novel conditional knockin Fbxw7R468C line should be useful to gain a more profound understanding of carcinogenesis associated with mutation of FBXW7.

FBXW7 (also known as FBW7, CDC4, SEL10, or AGO) is an F-box protein in the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complex that controls the degradation of target substrates via the ubiquitin-proteasome pathway. Most FBXW7 substrates are positive regulators of cell growth, including c-MYC1-2, Cyclin E3–5, c-JUN6, NOTCH7–9, KLF510,11, mTOR12, and MCL113,14. FBXW7 has therefore been characterised as a tumour suppressor in human cancer.

FBXW7 is located on chromosome 4q32, where deletions are frequently observed in human cancers. Analysis of the gene has revealed pathogenic mutations in approximately 6% of tumours. Frequent mutations have been reported in cholangiocarcinoma (35%), T-cell acute lymphocytic leukaemia (31%), and colon cancer (9%)15. Most of the mutations are missense mutations affecting arginine residues (Arg465, Arg479, and Arg505) within the WD40 domain, which is responsible for substrate recognition (Fig. 1A)16, and R465C is the mutation most frequently detected in human primary tumours (Catalogue of Somatic Mutations in Cancer; http://www.sanger.ac.uk/cosmic)16.

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A mouse line of Fbxw7R482Q corresponding to the FBXW7R479Q mutation in humans was previously established by Davis et al. This study reported that mice heterozygous for this mutation died perinatally due to a defect in lung development and other developmental abnormalities, such as eyelid fusion defect and cleft palate. In addition, an Fbxw7 R468C mouse line was previously generated and hematopoiesis was studied. Notably, it was reported that homozygous deletion of Fbxw7 caused embryonic lethality at E10.5–11.5 due to vascular abnormalities.

In this study, we established a novel mouse line carrying a conditional knockin allele of Fbxw7 R468C corresponding to FBXWR465C in humans. Although similar conditional knockin mouse lines with this mutation have been established and analysed by King et al., the effect of this mutation on mouse development has not been fully elucidated. We therefore generated mice with endogenous expression of Fbxw7 R468C, as well as mice whose liver tissues expressed heterozygous and homozygous Fbxw7 R468C alleles, and analysed their phenotypes.
of 338 bp. Lane 1: Fbxw7R468C/+; lane 2: Fbxw7R468C/−; lane 3: Fbxw7R468C/−, and lane 4: Fbxw7R468C/−; CAG-Cre. (B) PCR direct sequence analysis of RT-PCR products from Fbxw7R468C/− and Fbxw7120/+ mice. RNA was extracted from mouse embryonic fibroblasts (MEFs) isolated from E12.5 Fbxw7R468C/+ and Fbxw7R468C/− embryos.

Results and Discussion

Generation of transgenic mice containing a conditional knockin allele of the Fbxw7R468C mutation.

We used the Cre-LoxP system to generate transgenic mice containing a conditional knockin allele that expresses Fbxw7R468C only after Cre-mediated recombination. The targeting vector contained a left arm spanning exons 6–7 and a right arm spanning exons 8–10, which harboured two nucleotide changes, C1402T and G1404T (CGG to TGT), at codon 468 in exon 8 to express the Fbxw7R468C mutation (Fig. 1B). The arms were separated from each other by two LoxP sequences. Between these LoxP sites, we introduced a minigene cDNA encoding wild-type exons 8–11 preceded by a rabbit α-globin splice accepter site, followed by a polyadenylation sequence, and a right arm spanning exons 8–10, which harboured two nucleotide changes, C1402T and G1404T (CGG to TGT), at codon 468 in exon 8 to express the Fbxw7R468C mutation (Fig. 1B). The arms were separated from each other by two LoxP sequences. Between these LoxP sites, we introduced a minigene cDNA encoding wild-type exons 8–11 preceded by a rabbit α-globin splice accepter site, followed by a polyadenylation STOP sequence and the neomycin-resistant cassette (neo). Consequently, in the absence of Cre recombinase, the targeting vector expressed wild-type Fbxw7, including exons 8–11, in the minigene. The introduction of Cre recombinase induced deletion of the LoxP-STOP-LoxP (LSL) cassette and expressed Fbxw7R468C (Fig. 1B).

The vector was introduced into embryonic stem (ES) cells, and homologous recombined clones were identified using Southern blot analysis (Fig. 1C). Mice were generated from the positive clones, and a breeding colony was established in a C57BL/6 background. Genotyping by polymerase chain reaction (PCR) confirmed the inheritance of the Fbxw7R468C allele using DNA extracted from mouse tails (Fig. 1D). Although homozygous deletion of the Fbxw7 gene was reportedly lethal at E10.5–11.519,20, the homozygous mutant mice (Fbxw7−/−) were viable and fertile (Fig. 1D and S1) by the expression of wild-type Fbxw7 cDNA in the absence of Cre. No apparent developmental abnormalities were observed in either homozygous or heterozygous Fbxw7R468C/+ mice.

Expression of endogenous Fbxw7R468C in embryogenesis.

To investigate the effect of endogenous expression of the Fbxw7R468C mutation on mouse development, we next generated mice that permanently express Fbxw7R468C in all tissues. We used CAG-Cre mice that ubiquitously express Cre recombinase21. In progeny from heterozygous female CAG-Cre parents that possess a maternal effect, the target transgenes undergo complete excision of the floxed sequence in the embryos, even in the absence of CAG-Cre inheritance21. Thus, we crossed male Fbxw7R468C+/− mice to female CAG-Cre mice. Genotyping of the surviving offspring revealed that some newborns born with some dead offspring harboured the Fbxw7R468C allele. Additional genotyping of E18.5 embryos detected the successful recombination of the Fbxw7R468C allele irrespective of the inheritance of the CAG-Cre transgene, with an expected Mendelian ratio of 1:1 at E18.5 (Fig. 2A). Furthermore, Fbxw7R468C transcripts were confirmed in the Fbxw7R468C−/− embryos (Fig. 2B). At this time point, there were no obvious differences in the gross appearance of Fbxw7R468C−/− and wild-type embryos. Therefore, we concluded that the Fbxw7R468C−/− embryos died around birth. The results are consistent with the phenotype of the Fbxw7R468C−/− mutant embryos reported by Davis et al.22.

Developmental defects in Fbxw7R468C−/− mice.

To elucidate the cause of perinatal lethality of Fbxw7R468C−/− mice, we performed histological analysis of E18.5 embryos in both Fbxw7R468C−/− and wild-type mice. We first focused on the lungs in Fbxw7R468C−/− mice because defects in lung development have been reported in the Fbxw7120/+ mice. There were apparent differences in the lung histology of wild-type and mutant mice (Fig. 3A and B). Aerated lung areas in Fbxw7R468C−/− mice were small and their alveolar septa were thickened compared
with septa in wild-type littermates (Fig. 3A–D), suggesting that respiratory failure is the cause of perinatal lethality in \( Fbxw7^{R468C/+} \) mice. To clarify the cause of the lung abnormalities in E18.5 \( Fbxw7^{R468C/+} \) mice, cell proliferation was examined by immunohistochemical staining of Ki67 in the lung. We observed a significant increase in the number of Ki67-positive cells in the \( Fbxw7^{R468C/+} \) lung compared with the wild-type lung (Fig. 4A–C), which suggests that enhanced cell proliferation caused the thickening of the alveolar septa in the \( Fbxw7^{R468C/+} \) lung at E18.5.

Figure 3. Histological analysis of lungs in wild-type and \( Fbxw7^{R468C/+} \) mice. (A, B) Representative hematoxylin and eosin (H&E) staining of the lungs of wild-type (A) and \( Fbxw7^{R468C/+} \) (B) mice at E18.5. Bar, 50 \( \mu m \). (C) Aerated lung area in wild-type and \( Fbxw7^{R468C/+} \) mice at E18.5. (D) Thickness of the alveolar septum in wild-type and \( Fbxw7^{R468C/+} \) mice at E18.5. Error bars indicate the mean ± standard deviation (SD). *\( P < 0.05 \).

Figure 4. Cell proliferation in the lungs of wild-type and \( Fbxw7^{R468C/+} \) mice. (A, B) Immunohistochemical staining of Ki67 in the lungs of wild-type (A) and \( Fbxw7^{R468C/+} \) (B) mice. Bar, 50 \( \mu m \). (C) Quantification of Ki67-positive cells in wild-type and \( Fbxw7^{R468C/+} \) mutant mice. Error bars indicate the mean ± SD. *\( P < 0.05 \).
We also investigated other developmental defects, including eyes-open at birth (EOB) and cleft palate, in our Fbxw7R468C/+ mice model, since these defects were also previously reported in Fbxw7R482Q/+ mice\(^1\). EOB and cleft palate were consistently observed in 17 of 102 (17%) (Figure S2A–D) and one of 78 (1.3%) (Figure S2E and F) mice, respectively. However, the frequencies of these two abnormalities in Fbxw7 R468C/+ mice were lower than those reported in Fbxw7 R482Q/+ mice\(^1\). Although the difference in frequencies of the abnormalities may only reflect the difference in genetic background or housing conditions, it is possible that the functions of the R468C and R482Q mutants are not completely identical.

Since lung, eye, and palate abnormalities were not reported in Fbxw7+/– mice, the defects of these organs in Fbxw7R468C/+ mice, as well as Fbxw7R482Q/+ mice, are not due to the haploinsufficiency of Fbxw7, but rather due to its function as the ‘just enough’ or ‘dominant negative’ tumour suppressor proposed by Davis et al.\(^1\).

Effect of the Fbxw7R468C/+ mutation on substrate stability. Recent studies of FBXW7 have resulted in an increased number of its substrate proteins, including mTOR, NOTCH1, SREBP1, NF-κB2, MYC, KLF5, Cyclin E, c-JUN, MCL1, and TGIF1\(^2,3\). To investigate the effect of the Fbxw7R468C mutation on substrate stability, we analysed their expression levels using lysates of embryos and mouse embryonic fibroblasts (MEFs) from wild-type and Fbxw7R468C/+ mice by immunoblotting (Fig. 5A and B). Although the expression levels of Notch1, Tgif1, and Mcl1 were elevated in the lysates of Fbxw7R468C/+ embryos compared with those of wild-type embryos, no differences were observed in the levels of mTor, NF-κB2, Srebp1, Myc, Klf5, Cyclin E, and c-Jun (Fig. 5A).

Interestingly, the expression levels of Notch1, NF-κB2, Srebp1, Myc, Klf5, Cyclin E, and c-Jun were unchanged in the lysates of MEFs from Fbxw7R468C/+ mice compared with wild-type mice (Fig. 5A and B). In addition, real-time PCR analyses revealed no difference in the mRNA expression levels of some Fbxw7 substrates between MEF cells from Fbxw7R468C/+ mice and those from wild-type mice (Figure S3). These data suggest that the R468C mutation affects the stability of specific target proteins in a cell type-specific manner. In accordance with our observation, it has been reported that the Fbxw7R482Q mutation affects some of the reported Fbxw7 target proteins in a cell type-specific manner\(^1,2,4\).

The effect of liver-specific Fbxw7R468C knockin on biliary abnormalities and tumorigenesis. FBXW7 mutations are involved in various malignancies, including intrahepatic cholangiocarcinoma\(^3,5\). In addition, homozygous deletion of Fbxw7 in the murine liver reportedly induced hyperplasia of the bile duct and biliary hamartomas, also known as von Meyenburg complexes\(^6\). Therefore, we next investigated the livers of conditional Fbxw7R468C knockin mice. We crossed Fbxw7\(\text{LSL-R468C/+}\) mice with Alb-Cre mice, and generated Alb-Cre;Fbxw7\(\text{LSL-R468C/+}\) mice, whose liver tissues expressed...
the heterozygous and homozygous Fbxw*R468C allele, respectively. These mice were alive and did not show obvious developmental defects. There were no apparent abnormalities in the livers of Fbxw7Hep-R468C/+ mice until 12 months of age (Fig. 6A and B). In contrast, dilatation and hyperplasia of bile ducts were observed throughout the livers of Fbxw7Hep-R468C/R468C mice at the age of 12 months (Fig. 6C). Consistent with the report by Onoyama et al., our data imply that wild-type Fbxw7 protein is necessary for the development of a normal bile duct.

We further assessed the expression of Fbxw7 target proteins in the livers of control (Alb-Cre), heterozygous (Fbxw7Hep-R468C/+), and homozygous (Fbxw7Hep-R468C/R468C) knockin mice by immunoblot analysis. We did not observe a significant difference in the expression of target proteins between heterozygous (Fbxw7Hep-R468C+/+) and control mice (Fig. 6D); however, the expression of Myc, Klf5, and c-Jun was markedly induced in the livers of homozygous mice (Fbxw7Hep-R468C/R468C). In addition, the expression of mTor, Srebp1, and Mcl1 was modestly increased in the livers of homozygous mice (Fig. 6D). These data suggest that the accumulation of these proteins may be involved in the abnormal development of the bile duct in Fbxw7Hep-R468C/R468C mice.

To further investigate the role of the Fbxw7R468C mutation in murine liver tumourigenesis, we introduced a liver-specific Fbxw7R468C mutation in combination with an oncogenic Kras mutation. Mice carrying the conditional knockin allele of the KrasG12D mutation (KrasLSL-G12D/+) were crossed with Alb-Cre mice and Fbxw7LSL-R468C/+ mice to generate Alb-Cre;KrasLSL-G12D/+(Fbxw7LSL-R468C/+) and Alb-Cre;KrasLSL-G12D/+(Fbxw7LSL-R468C/+) mice. Although no abnormal histological findings were detected in Alb-Cre;KrasLSL-G12D/+(Fbxw7LSL-R468C) mice, carrying the liver-specific KrasG12D alone, until 8 months after birth, the heterozygous Fbxw7LSL-R468C mutation in combination with the oncogenic Kras mutation induced bile duct dilation and hyperplasia in some mice at the age of 8 months (Fig. 7A and B). Furthermore, the homozygous Fbxw7LSL-R468C mutation in combination with oncogenic Kras induced cholangiocarcinoma-like lesions composed of dysplastic dust-like structures surrounded by fibrosis in all mice within 2 months of birth (Fig. 7C). These findings suggest the effects of the Fbxw7R468C mutation in the promotion of cholangiocarcinogenesis. Considering that the majority of FBXW7 mutations in human cancer are heterozygous and a previous study revealed the promotive effects of intestinal polyps by the heterozygous Fbxw7R482Q mutation on an Apc
mutant background24, further studies are required to follow up the phenotypes of the liver-specific heterozygous Fbxw7R468C mutant mice with or without oncogenic Kras mutation.

Collectively, we successfully established a novel mouse line that conditionally and endogenously expresses the Fbxw7R468C mutant, the mouse counterpart of human FBXW7R468C. Mutant mice showed several developmental abnormalities, including lung defects, EOB, and cleft palate. Furthermore, endogenous expression of the Fbxw7R468C mutant affected a subset of reported target proteins, such as Notch1 and Tgif1, in a tissue-dependent manner. In addition, homozygous expression of mutant Fbxw7R468C induced dilatation of the bile duct in the murine liver, which was accompanied by the remarkable augmentation of Myc, Klf5, and c-Jun proteins. Furthermore, homozygous expression of this mutant cooperated with oncogenic Kras to induce intrahepatic cholangiocarcinoma-like lesions. This conditional knockin mouse line of the cancer-associated hotspot mutation of FBXW7 should therefore be a useful tool for elucidating the mechanisms underlying human neoplasms that are associated with FBXW7 mutation.

Materials and Methods

Generation of the conditional Fbxw7R468C allele. Conditional Fbxw7R468C knockin mice were generated by gene targeting. The targeting vector was constructed by cloning of the left, middle, and right arms in the pEz-Frt-loxP-DT vector. Both fragments of the left (4.4 kb) arm containing exons 1–7, and the right (4.9 kb) arm containing exons 8–11 were amplified by PCR using bacterial artificial clone RP23-269G19 as a template. Two mutations (C1402T and G1404T) were introduced into exon 8 on the right arm using site-directed mutagenesis. Additionally, a fragment of cDNA consisting of exons 8–11 was amplified using a gene-specific primer set containing a rabbit α-globin splice acceptor at the 5' end and a polyadenylation signal at the 3' end with wild-type Fbxw7 cDNA as a template. The PCR product was subsequently cloned into two loxP sites (black triangles) of the pEz-Frt-loxP-DT vector using the In-Fusion HD Cloning System (TAKARA) according to the manufacturer's instructions. The minigene containing exons 8–11 of Fbxw7 cDNA and the Neo gene flanked by two FRT sites (grey triangles) in the LSL cassette were cloned between the left and right arms of the targeting vector. The construct was electroporated into EGR-101 ES cells and G418-resistant clones were selected. Homologous recombinant ES clones were screened by Southern blotting using the probes as indicated in Fig. 1B. Positive clones were injected into mouse C57BL/6 blastocysts and chimeric mice were generated by an aggregation method28. Chimeric male mice were bred to female mice to achieve germline transmission. Male Fbxw7R468C/+ mice were crossed with female Alb-Cre (Jackson Laboratory) mice. Alb-Cre;KrasLSL-G12D/+ Fbxw7LSL-R468C/LSL-R468C mice at the age of 8 weeks (B) and Alb-Cre;KrasLSL-G12D/+ Fbxw7LSL-R468C/LSL-R468C mice at the age of 8 months (A) and Alb-Cre;KrasLSL-G12D/+ Fbxw7LSL-R468C/LSL-R468C mice at the age of 8 weeks (C). Bar, 100μm.

Figure 7. Liver histology in mice with heterozygous and homozygous Fbxw7R468C mutations in combination with an oncogenic KrasG12D mutation. (A–C) Representative H&E staining of the livers of Alb-Cre;KrasLSL-G12D/+ (A) and Alb-Cre;KrasLSL-G12D/+ Fbxw7LSL-R468C/LSL-R468C mice at the age of 8 months (B) and Alb-Cre;KrasLSL-G12D/+ Fbxw7LSL-R468C/LSL-R468C mice at the age of 8 weeks (C). Bar, 100μm.
Detection of the Fbxw7R468C mutation. For analysis of the Fbxw7 mutations in genomic DNA, a region encompassing codon 468 was amplified using DNA from the tail tips and the following set of primers: Fbxw7-D/R-F (5'-GTGAGCAGATAAGAGGACA-3') and Fbxw7-D/R-R (5'-CTATAGATCCAGAGTCACA-3'). For analysis of the Fbxw7 mutations in mRNA, another region encompassing the cDNA was amplified using cDNA from MEFs and the following set of primers: Fbxw7-D/R-F and Fbxw7-D/R-R (5'-CATCGAGCTGTAACACT-3'). cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) with 1 μg of total RNA extracted from MEFS using an RNeasy Mini Kit (Qiagen). PCR reactions were performed using KOD Plus DNA Polymerase (Toyobo) with 35 cycles at 98 °C for 10 sec, 60 °C for 30 sec, and 68 °C for 30 sec. To confirm the sequence, PCR products were purified using a PCR purification kit (Qiagen) and sequenced on an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific) with the BigDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific). The Fbxw7-D/R-F primer was used for sequencing.

Histology and immunohistochemistry. E18.5 embryos were collected, fixed with 10% formalin, and embedded in paraffin for sectioning. Sections (3 μm) were stained with hematoxylin and eosin. For immunohistochemistry, sections were dewaxed in xylene and rehydrated by immersion in 100% ethanol and distilled water. Antigen retrieval was performed by boiling for 10 min in 10 mM citrate solution (pH 6.0). Sections were incubated for 10 min in 0.3% H2O2-methanol for blocking endogenous peroxidase, blocked with 10% serum for 30 min at room temperature, and incubated overnight at 4 °C with anti-Ki67 antibodies (1:50; Clone TEC-3, DAKO). Slides were rinsed in phosphate-buffered saline and peroxidase activity was detected using the Vectastain Elite ABC Kit (Vector Laboratories) and ImmPACT DAB substrate (Vector Laboratories). Nuclei were counterstained with hematoxylin for 1 min.

Immunoblot analysis. Lysates were extracted from the heads of E12.5 embryos, MEFS, and adult livers in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) supplemented with a Protease Inhibitor Cocktail Set III (Calbiochem). Immunoblot analysis was performed using the following primary antibodies: mTOR (7C10, Cell Signaling), Notch1 (D1E11, Cell Signaling), NF-κB (2A4, Novus Biologicals and H-160, Santa Cruz), and β-actin (AC-15, Sigma). Each primary antibody was used at a 1:1000 dilution. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (GE Healthcare) was used as the secondary antibody. Immunoreactive proteins were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions.

Real-time PCR. Total RNA from MEFS was extracted with Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time PCR was performed using the qPCR Kapa SYBR Fast ABI Prism Kit (Kapa Biosystems) on StepOnePlus (Thermo Fisher Scientific). The amount of transcript was determined using the relative standard curve method, and Gapdh was used as the internal control. The primer sequences used are shown in Table S1.

Statistical analysis. Statistical significance between the two groups was determined using Student’s t-test. Data are displayed as the mean ± standard deviation. Probability values less than 0.05 were considered statistically significant.

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Author Contributions
T.I. conceived the study. T.I. and Y.T. designed the experiments. T.I., Y.T., C.Z., L.X., and T.O. conducted the experiments. T.I., Y.T., and D.M. conducted histopathological examinations. S.K., S.K., and Y.I. performed ES cell targeting and blastocyst injection. T.S. assisted T.I. and T.F. in designing and constructing the targeting vector. T.I., Y.T., K.Y., and Y.F. analysed the data and discussed the results. T.I., Y.T., and D.M. conducted histopathological examinations. T.I. and T.F. designed and constructed the targeting vector. S.K., S.K., and Y.I. performed ES cell targeting and blastocyst injection. T.S. assisted T.I. and T.F. in designing and constructing the targeting vector. T.I., Y.T., K.Y., and Y.F. analysed the data and discussed the results. T.I., Y.T., and Y.F. wrote the manuscript.

Additional Information
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