Investigation of the atypical FBXW7 mutation spectrum in human tumours by conditional expression of a heterozygous propellor tip missense allele in the mouse intestines

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ABSTRACT

Objective FBXW7 encodes the substrate recognition component of a ubiquitin ligase that degrades targets such as Notch1, c-Jun, c-Myc and cyclin E. FBXW7 mutations occur in several tumour types, including colorectal cancers. The FBXW7 mutation spectrum in cancers is unusual. Some tumours have biallelic loss of function mutations but most have monoallelic missense mutations involving specific arginine residues at β-propellor tips involved in substrate recognition.

Design FBXW7 functional studies have generally used null systems. In order to analyse the most common mutations in human tumours, we created a Fbxw7(R482Q+)/+ mouse and conditionally expressed this mutation in the intestines using Vill-Cre. We compared these mice with heterozygous null (Fbxw7−/-) mutants.

Results A few sizeable intestinal adenomas occurred in approximately 30% of R482Q/+ and Fbxw7−/- mice at age >300 days. Breeding the R482Q allele onto Apc mutant backgrounds led to accelerated morbidity and increased polyp numbers and size. Within the small bowel, polyp distribution was shifted proximally. Elevated levels of two particular Fbxw7 substrates, Klf5 and Tgif1, were found in normal intestine and adenomas of ApcR482Q/+ mice, but not Fbxw7−/- animals. On the Apc mutant background, Fbxw7−/- mutants had a phenotype intermediate between fbxw7 wild-type and R482Q/+ mice.

Conclusions Heterozygous Fbxw7 propellor tip (R482Q) mutations promote intestinal tumorigenesis on an Apc mutant background. Klf5 and Tgif1 are strong candidates for mediating this effect. Although heterozygous null fbxw7 mutations also promote tumour growth, these have a weaker effect than R482Q. These findings explain the FBXW7 mutation spectrum found in human cancers, and emphasise the need for animal models faithfully to reflect human disease.

OBJECTIVE

FBXW7 (F-box and WD40 repeat domain containing 7, also known as FBW7, hCDC4), a tumour suppressor gene, is mutated in many human malignancies.1 FBXW7 encodes the substrate recognition component of a Skp, Cullin, F-box-containing (SCF)-E3 ubiquitin ligase complex and negatively regulates multiple proteins with established roles in

Significance of this study

What is already known about this subject?

▸ FBXW7 is commonly mutated in tumours of diverse origins, including colorectal cancer.

▸ FBXW7 is classed as a tumour suppressor, but has an unusual mutation spectrum whereby biallelic, simple loss-of-function mutations are rare; instead, most mutations are monoallelic missense changes involving specific arginine residues at β-sheet propellor tips that allow the FBXW7 protein to recognise its substrates.

▸ To date, mouse models carry Fbxw7 null alleles, but these do not faithfully recapitulate the mutations most commonly present in human cancers.

What are the new findings?

▸ Conditional expression of a heterozygous propellor tip missense Fbxw7 allele in the mouse intestines causes tumorigenesis.

▸ The mutation causes elevated levels not of classical Fbxw7 substrates such as c-Jun, but of Klf5 and Tgif1, in both normal intestines and adenomas.

▸ On an Apc mutant background, heterozygous null Fbxw7 mutations promote tumour growth, but have a much weaker effect than heterozygous propellor tip mutants.

▸ Propellor tip mutations most likely act as dominant negative, loss-of-function alleles that provide sufficient derangement for tumorigenesis and they are found commonly because they require only a single ‘hit’.

How might it impact on clinical practice in the foreseeable future?

▸ Use of specific animal models can explain the in vivo mutation spectrum of cancer genes.

▸ Genetically targeted therapies need to take into account the specific mutations in cancer and how they act.

▸ FBXW7 substrates exhibit tissue specificity; here, Tgf1 and Klf5 have been identified as the strongest candidate substrates for therapeutic intervention.
the control of cell division and growth, including cyclin E, c-Jun, c-Myc, mTor and Notch. There are several excellent reviews of the growing knowledge of FBXW7.2–5

As it became apparent that FBXW7 regulates many oncoproteins, it was hypothesised that mutations in FBXW7 may cause tumorigenesis. FBXW7 mutations were initially identified by Spruck et al in ovarian and breast cancer cell lines.6–7 Since then many studies have assessed FBXW7 mutation status in a range of cancer types, including both solid tumours and haematological neoplasms. A study by Akhoondi et al8 significantly contributed to this collection, as extensive genetic screening of over 500 primary tumours of diverse tissue origins was carried out, identifying mutations in FBXW7 in 6% of tumours. Variations in mutation frequencies among these tissues were seen, with the highest frequencies found in lesions from the bile duct, endometrium, blood and colorectum.

FBXW7 has several important domains, including WD40 repeats that form an eight-bladed, barrel-shaped β-propeller (see online supplementary figure S1A) which provides a binding pocket for substrates.10 11 Critical arginine residues at the apex of the propellers directly interact with ‘destruction recognition’ (CDC4 phosphodegron, CPD) sequences in substrates.10 11 The Catalogue of Somatic Mutations in Cancer (COSMIC) database reports 496 mutations in FBXW7 across all tissue types; 53% of these are missense changes affecting arginine residues 465, 479 and 505 that lie at the β-propeller tips which interact with FBXW7 substrates, with the majority of these propeller tip mutations being monoallelic.

FBXW7 mutations have been investigated thoroughly in colorectal tumours.12–14 In colorectal cancer, 189 mutations have been reported, of which 44% are missense mutations at amino acids 465, 479 and 505. We have analysed The Cancer Genome Atlas (TCGA) set of 226 colorectal cancers and find no evidence that arginine propeller tip missense mutations are associated with clinicopathological variables, including gender, age of presentation and cancer stage (details not shown). Non-propeller tip mutations are found more frequently in hypermutated cases (p=0.04), suggesting that some of these mutations are background changes.

Loss of heterozygosity at FBXW7 is observed infrequently, especially for WD40 propeller tip mutations, and few tumours carry biallelic FBXW7 mutations predicted to abolish protein function.13 15 These data suggest that FBXW7 does not function as a standard tumour suppressor gene through the two hit mechanism of inactivation,16 and partial loss of FBXW7 function may be sufficient to cause tumorigenesis. We have formalised this suggestion in the ‘just enough’ model15 whereby protein-inactivating monoallelic (heterozygous) FBXW7 mutations provide insufficient functional derangement to drive tumour growth, although they can do so as homoyzogotes or compound heterozygotes. However, monoallelic propeller tip mutations cause an intermediate level of functional derangement that is sufficient to promote tumorigenesis.

Two groups have previously generated simple knockout mice carrying null Flxw7 alleles.17 18 In the heterozygous mutant state, these mice appeared normal with no tumour formation up to 1 year of age. However, null homoyzogotes suffered embryonic lethality at E10.5–11.5 due to compromised cardiovascular development that was attributed to elevated Notch1 expression.17 18 Subsequently, conditional Fbxw7-null mice were used to study Fbxw7 in T cell,19 haematopoietic stem cell20 21 and gut lineages.22 23 Sancho et al22 showed that wild-type Flxw7 mRNA was highly expressed in the transit amplifying/progenitor cell compartment of the intestines, with a threefold increase in

the crypt compared with the villus.22 Homozygous Fbxw7 knockout resulted in impaired intestinal cell differentiation, with a reduction in the number of goblet and Paneth cells. There was an accumulation of intracellular domain of Notch1 (Nicd1) and phosphorylated c-Jun. Heterozygous Fbxw7 knockout resulted in an accumulation of Nicd1, but not phospho-c-Jun. Notch is a key regulator in the binary decision of transit amplifying cells to differentiate into secretory or absorptive cells and therefore elevated Nicd1 protein levels in Fbxw7 null cells were reported as the likely cause of the differentiation phenotype observed. Despite the accumulation of actively proliferating progenitor cells in the guts of homozygous null mice, no intestinal tumours were reported up to 11 months of age. Another group23 subsequently studied the same conditional Fbxw7 null mouse, finding a small number of adenomas in the large intestines of homozygous knockout animals at 9–10 months of age. Increased crypt budding and fission, but no polyps, were observed in the small intestine.

As the Fbxw7 null mutant mice do not faithfully recapitulate the mutations most commonly present in human cancers, we have generated a conditional Fbxw7 mutant mouse which carries one of the most commonly occurring propellor tip missense mutations. We have used this to determine the consequences of propellor tip mutations, and compared the phenotypic effects of this mutation with those of heterozygous null alleles, both in the normal intestine and in intestinal polyps.

**DESIGN**

**Generation and genotyping of R482Q mice**

Generation and genotyping of Fbxw7fl/fl R482Q/+ mice were described previously24 (see online supplementary figure S1B). Fbxw7fl/fl R482Q/+ mice were crossed with Vill-Cre mice25 in order to knock-in the R482Q mutation (equivalent of human R479Q) in the intestine. These mice shall be referred to as R482Q/+ in the heterozygous state. Conditional Fbxw7 null animals22 were crossed with Vill-Cre animals; these will be referred to as Fbxw7fl/fl R482Q/+ in the heterozygous state. Two types of Apc mutant mice, ApcMin/+ (Min)26 and ApcMin+/+ (1322T)27 were also used in this study. All procedures were carried out in accordance with Home Office UK regulations and the Animals (Scientific Procedures) Act 1986. All mice were housed at London Research Institute, Clare Hall Laboratories, Cancer Research UK or Functional Genomics Facility, Wellcome Trust Centre for Human Genetics, Oxford University. All strains used in this study were maintained on the C57Bl/6J background for ≥6 generations.

**Tissue preparation and histology**

Mice were sacrificed when showing symptoms of intestinal polypos (anaemia, hunching) by cervical dislocation. The intestinal tract was removed immediately and divided into small intestine (proximal/SB1, middle/SB2 and distal/SB3) and large intestine. The intestines were opened longitudinally, using a gut preparation apparatus,28 washed in phosphate buffered saline (PBS) and fixed overnight in 10% neutral buffered formalin. For scoring of polyps, gut preparations were stained with 0.2% methylene blue for 10 s, washed in PBS for 20 min and polyps were counted/measured using a dissecting microscope at 3× magnification. Fixed specimens were embedded and haematoxylin–eosin stained following standard protocols.

**Western blotting**

For intestinal tissue, isolation of epithelial cells was carried out. A 2 cm long piece of intestine was opened longitudinally and

incubated in PBS supplemented with 30 mM EDTA for 2 h with agitation at 4°C. Shed epithelial tissue was pelleted and lysed in RIPA buffer with the addition of protease inhibitors (Complete Mini, Roche) on ice for 30 min. Nuclear fractions were generated as described previously.24 Lysates were quantified using the BCA assay (Thermo Scientific). Western blotting was performed following standard methods using the following antibodies: Cyclin E (sc-481, Santa Cruz), c-Jun (sc-44, Santa Cruz), c-Myc (sc-47694, Santa Cruz), p-c-Myc (Thr58/Ser62) (sc-8000R, Santa Cruz), Klf5 (ab24331, Abcam), Tgif1 (H-171) (sc-9084, Abcam), activated notch 1 (Nicol1) (ab8925, Abcam), Lamin B1 (H-90 sc-20682, Santa Cruz), FLAG (F1804, Sigma-Aldrich) and c-Myc epitope tag (S1826, Clontech). Bands were quantified using pixel intensity normalised to loading control (ImageJ).

Immunohistochemistry
Immunohistochemistry for performed as described previously using the following antibodies: alkaline phosphatase (ab65834, Abcam), β-catenin (610154, BD), caspase3 (AF835, R and D systems), chromogranin A (sc-20200, Santa Cruz), Ki67 (sc-7579, Santa Cruz), Tgif1 (sc-9084, Abcam), activated notch 1 (Nicol1) (ab8925, Abcam), Lamin B1 (H-90 sc-20682, Santa Cruz), FLAG (F1804, Sigma-Aldrich) and c-Myc epitope tag (S1826, Clontech). Bands were quantified using pixel intensity normalised to loading control (ImageJ).

Co-immunoprecipitation of tagged Fbxw7 plasmids
Murine wild-type and R482Q Fbxw7 coding region cDNAs were cloned into pCMV Myc and FLAG tagged mammalian expression plasmids, respectively (Clontech). Plasmids were transiently transfected into RKO cells and co-immunoprecipitated using protein G Dynabeads (Invitrogen) and 2 μg of Myc antibody (S1826 Clontech) and anti-FLAG M2 magnetic beads (Sigma-Aldrich) for the Myc and FLAG co-immunoprecipitation, respectively.

Expression of exogenous wild-type FBXW7 in colorectal cancer cell lines
The FBXW7 overexpression plasmid, p3xFLAG-Myc-CMV-24, containing human FBXW7α isoform (pFLAG FBXW7α) was kindly provided by Bruce Clurman (Fred Hutchinson Cancer Research Center, Seattle). The same vector lacking the FBXW7α insert was used as an empty vector control (pEV).

shRNA knockdown of FBXW7
pLKO.1 short hairpin RNA (shRNA) lentiviral clones TRCN0000006556 (6) and TRCN0000006557 (7) (Open Biosystems) were used to knockdown FBXW7 expression. Lentiviral particles were packaged following standard protocols and titrated using the Lenti-X p24 Rapid Titre Kit (Clontech). LOVO cells were transduced with 100 ng of packaged virus. Efficient knockdown of FBXW7 mRNA (~75%) was detected by quantitative real-time PCR using TaqMan probe Hs00217794_ml (Applied Biosystems).

Additional methods
Additional methods are provided in the online supplementary data.

RESULTS
Generation of intestine-specific R482Q and Fbxw7 null mutant mice
To ascertain whether Fbxw7 propellar tip mutations could induce intestinal tumorigenesis, Fbxw7Δ(ex5ΔΔ)/(fl/fl) mice were bred with Vill-Cre25 animals to give Vill-Cre:Fbxw7Δ(ex5ΔΔ)/(fl/fl) (R482Q/+) mice. These mice express Cre recombinase in all cell types of the intestinal epithelium29 and we confirmed knock-in of the R482Q mutation along the entire intestine (see online supplementary figure S1C, D). Similarly, for our comparison group, we crossed conditional Fbxw7Δ(ex5ΔΔ)/+ (Fbxw7+/−) animals and confirmed efficient Cre-mediated recombination as above.

Normal intestines of Fbxw7 mutant mice show subtle alterations in cell lineages and elevated levels of apoptosis
To ascertain whether molecular defects were present in normal intestinal tissue in young Fbxw7 mutant mice, we examined a range of cell lineage markers. There were no significant differences in crypt size or the positions of specialised cell types among mice (details not shown). The number of goblet cells per villus, which varied from seven to 11 across all mice (see online supplementary figure S2), showed an R482Q dosage-dependent reduction in numbers compared with wild-type (p<0.001, non-parametric trend test), in agreement with previous findings in Fbxw7+/− and Fbxw7+/+ animals25 that we also replicated here (details not shown). There were no significant differences in any of the other cell lineages (see online supplementary figure S2) and no change in the size of the stem cell compartment, as assessed using Lgr5 and Olfm4 mRNA expression.

As many Fbxw7 substrates are critical proteins in proliferation and apoptosis, these processes were assessed in morphologically normal intestinal tissue from Fbxw7 mutant mice and controls. No significant difference in proliferation was observed (see online supplementary figure S3A, B). However, there were significant dosage-dependent increases in apoptosis both in R482Q/+ and R482Q/R482Q animals (p<0.001, non-parametric trend test), and in Fbxw7+/− and Fbxw7+/+ animals (p=0.010) compared with wild-type (see online supplementary figure S3A, C). There was no significant difference in apoptosis between the R482Q and Fbxw7 null alleles (p=0.138, ANOVA).

Normal intestine from R482Q/+ mice, but not Fbxw7+/− animals, shows elevated levels of Fbxw7 substrates Klf5 and Tgif1
As Fbxw7 regulates a plethora of substrates, we selected for analysis those known to be oncproteins or involved in intestinal development or homeostasis. We found no significant difference in the protein levels of Aurora A, Nicd1, c-Myc, p-c-Myc or c-Jun between wild-type, R482Q/+ and R482Q/R482Q mice (see online supplementary figure S4). Elevated levels of cyclin E were found in normal R482Q/R482Q tissue compared with wild-type controls (p=0.003, t test), although no significant increase in protein expression was seen in R482Q/+ tissue (figure 1). Two substrates, Tgif1 and Klf5, showed elevated levels in both R482Q/+ and R482Q/R482Q mice (for Tgif1, p=0.003 and p=0.001, respectively; for Klf5, p=0.036 and p=0.010, respectively; t test). The trend towards increased substrate levels with R482Q dosage was significant in both cases (for Tgif1, p=0.002 and for Klf5, p=0.048, non-parametric trend test). However, there were no significant differences in
Tgif1 or Klf5 levels between R482Q/+ and R482Q/R482Q mice (p=0.547 and p=0.567, respectively, t test).

Fbxw7 substrates were then assessed in Fbxw7+/− and Fbxw7−/− mice (figure 1, see online supplementary figure S4). For both Tgif1 and Klf5, we found no increase in protein levels over wild-type in Fbxw7−/− animals (for Tgif1, p=0.440 and for Klf5, p=0.433, t test). However, significant increases were found in Fbxw7−/− animals (for Tgif1, p=0.002 and for Klf5, p=0.001, t test). No dosage-dependent elevation in levels was found (p=0.064 for Tgif1 and p=0.060 for Klf5, non-parametric trend test), and there were significant differences in Tgif1 and Klf5 levels between Fbxw7−/− and Fbxw7+/− mice (p=0.007 and p=0.008, respectively, t test).

Finally, we compared Tgif1 and Klf5 across the R482Q− and Fbxw7−/− mice. The level of Tgif1 protein in R482Q+/+ tissue was significantly higher than in Fbxw7−/− tissue (p=0.026, t test). The level of Klf5 was similarly higher in R482Q+/+, although this did not reach formal significance (p=0.105). There were no significant differences in Tgif1 and Klf5 levels among R482Q+/+, R482Q/R482Q and Fbxw7−/− tissues (p>0.45 in all cases, t test).

**Intestinal tumours occur in aged R482Q/+ and Fbxw7−/− mice**

R482Q/+ mice developed no intestinal polyps or other gross defects up to 215 days of age (n=8). However, a R482Q/+ mouse showed signs of distress at 409 days old and was found to carry a large polyp in the small intestine (see online supplementary figure S5). We then aged a cohort of 41 R482Q/+ mice to >300 days old. Twelve (29%) mice exhibited a low level of Tgif1 expression was observed, comparable with polyps from 1322T mice.27 We then screened 11 R482Q/+ polyps for somatic mutations in Apc (exons 15), Ctnnb1 (exon 2), Tp53 (exons 5–8) and Kras (exons 1–2). Two Ctnnb1 p.Thr41Ile mutations were found (see online supplementary figure S6) in polyps from different mice. This is a known driver mutation in human colorectal tumours. We found no loss of heterozygosity of Fbxw7 in R482Q/+ tumours (n=4). To test for other chromosomal deletions or gains, array comparative genomic hybridisation was carried out on DNA extracted from the R482Q/+ adenocarcinoma, but no copy number variants were identified.

**Adenomatous polyps from R482Q/+ mice exhibit activation of the Wnt signalling pathway**

The late age of presentation of tumours and incomplete penetrance of phenotype in R482Q/+ mice suggested that accumulation of additional mutations was necessary for tumour development. As the majority of intestinal polyps acquire activated Wnt signalling, we assessed this pathway by investigating localisation of β-catenin protein using immunohistochemistry (figure 2). In general, cytoplasmic and low level nuclear β-catenin expression was observed, comparable with polyps from 1322T mice.27 We then screened 11 R482Q/+ polyps for somatic mutations in Apc (exons 15), Ctnnb1 (exon 2), Tp53 (exons 5–8) and Kras (exons 1–2). Two Ctnnb1 p.Thr41Ile mutations were found (see online supplementary figure S6) in polyps from different mice. This is a known driver mutation in human colorectal tumours. We found no loss of heterozygosity of Fbxw7 in R482Q/+ tumours (n=4). To test for other chromosomal deletions or gains, array comparative genomic hybridisation was carried out on DNA extracted from the R482Q/+ adenocarcinoma, but no copy number variants were identified.

**R482Q enhances intestinal polyposis in Apc mutant mice**

Multiple mutations are necessary for carcinogenesis, and as APC mutations occur in a large majority of human colorectal cancers, FBXW7 mutations are found mostly in the context of APC mutant alleles. To address whether Fbxw7 propellor tip mutant protein could promote the development of intestinal polyposis initiated by Apc mutations, R482Q mice were bred with two Apc mutants, Min26 and 1322T.23 R482Q/+; Apc compound mutant (test) and Fbxw7−/− wild-type; Apc mutant (control) mice were aged until test mice were symptomatic. Control littersmates were then sacrificed alongside the test mice. Although multiple adenomatous polyps were present in all animals, invasive tumours were not detected in either R482Q/+;Min or R482Q/+;1322T mice—this may be a result of the shortened lifespan of mice due to tumour burden, which prevents the accumulation of the somatic mutations necessary for tumour progression.
R482Q/+;Min mice were sacrificed at 87–111 days (see online supplementary figure S7). There was a significantly greater number of tumours in the R482Q/+;Min mice than the Min mice (p=0.002, t test; see online supplementary figure S7A). Both types of mouse displayed most polyps in SB3 (see online supplementary figure S7B). Polyps also tended to be larger in the double mutant animals (see online supplementary figure S7C); in Min animals, the majority of polyps were <1 mm in diameter, whereas in R482Q/+;Min compound mice, most were 1–2 mm in diameter (p=0.0135, t-test).

The equivalent comparison was then performed with 1322T rather than Min mice. R482Q/+;1322T animals showed signs of an intestinal phenotype at 71–127 days. There was a modest, non-significant increase in total tumour numbers in R482Q/+;1322T mice compared with 1322T controls (p=0.769, t test; see online supplementary figure S7A). R482Q/+;1322T polyps showed the same distribution as 1322T polyps, with the heaviest burden in SB1 (see online supplementary figure S7B), and the presence of the R482Q mutation had no significant impact on tumour size (p=0.651; see online supplementary figure S7C).

A combined stratified analysis of the effects of R482Q in both Min and 1322T mice was then performed. This showed that total number (p=0.0075, ANOVA) and size (p=0.011, logistic regression) of polyps was increased by the presence of the R482Q mutation (see online supplementary figure S7A). Only two R482Q/1322Q; Apc mutant mice were available for analysis, but these showed significantly more and larger polyps than Fbxw7 wild-type mice (p=0.0014 and p=0.012, respectively).

The levels of selected Fbxw7 substrates were analysed in R482Q/+;1322T and 1322T tumours paired for size and location. There was no significant difference in the levels of c-Jun, Aurora A, Nicd1, cyclin E or p-c-Myc between the two types of mice (p > 0.05 in all cases, t test; see online supplementary figure S8). However, levels of Kl5 and Tgf1 were elevated in

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**Figure 2**  Proliferation and Wnt activation in representative R482Q/+ polyps shown by immunohistochemistry. (A) haematoxylin–eosin stained section of polyp—scale bars 300 μm. (B) Ki67 expression in polyp—scale bars 300 μm. (C, D) β-Catenin expression—scale bars 150 μm; arrowheads highlight nuclear staining.

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**Figure 3** Elevated levels of Kl5 and Tgf1 protein in R482Q/+;Apc mutant tumours. The graph shows the ratio of average normalised pixel intensities, relative to 1322T tumours. The hyperphosphorylated (hyperp) band of Tgf1 was quantified, consistent with the paradigm that Fbxw7 substrates are phosphorylated prior to ubiquitination by Fbxw7. Results represent two technical replicates.
not significantly influenced by the presence of the monoallelic null allele. Furthermore, shRNA-mediated 75% knockdown of FBXW7 mRNA in colorectal cancer lines with propeller tip mutations produced no significant increase in TGF1 levels (figure 5C), suggesting that the monoallelic mutation produced a strong functional effect that could not readily be enhanced in the system used. Overall, while not conclusive on their own, these data support our mouse data showing a significant functional difference between monoallelic propeller tip Fbxw7 mutations and the heterozygous null genotype.

CONCLUSIONS

We have demonstrated that an Fbxw7 propeller tip mutation at arginine 482—a site homologous to amino acid 479 that is commonly mutated in human tumours—directly promotes intestinal tumorigenesis in a mouse model. It is likely that this must occur on a background of increased Wnt signalling, such as that provided by germline Apc mutations, or by spontaneous somatic mutation of genes such as β-catenin in older animals.

We compared our Fbxw7R482Q/+ (R482Q(+)) mice with the Fbxw7 null mice of Sancho et al.22 bred in the same facility. The principal comparison was to test our hypothesis that the R482Q allele occurs commonly in human tumours because it provides a greater level of functional derangement than a single null Fbxw7 allele. On an Apc mutant background, polyposis developed more rapidly in R482Q than Fbxw7−/− animals, and there was additionally a relative shift in polyp numbers to SB1, a feature previously associated with more severe disease.30 32

Of the Fbxw7 substrates tested, only Tgif1 and Klf5 protein levels were consistently increased in R482Q mutant normal tissues and in polyps. Fbxw7−/−—but importantly not Fbxw7+/−—animals also showed a significant change in Tgif1 and Klf5 expression. KLF5 is a zinc finger family transcription factor that plays a role in multiple cellular processes, including cell cycle regulation, proliferation and angiogenesis.33 It is primarily expressed in proliferative regions of the intestinal epithelium,34 35 and most data suggest that it promotes G1–S phase transition.34 35 We found no significant changes in proliferation in morphologically normal tissue from R482Q mutant mice but it has been shown31 36 that Klf5+/− mice have a greatly reduced intestinal tumour load. TGF1 is a negative regulator of the transforming growth factor β (TGF-β) signalling pathway, acting as a co-repressor in Smad2/3 complexes.37 Although there is a complex relationship between TGF-β activity and carcinogenesis, TGF-β component inactivation is common in colorectal tumours, and increased TGF1 may therefore contribute to intestinal tumorigenesis in our mice. To date, however, no direct assessment has been performed of the effects of Tgif1 mutation on intestinal tumours.

We additionally found that cyclin E levels were increased in mice homozygous for the R482Q or null mutation. However, Nicd1 levels were not significantly raised in Fbxw7−/− or Fbxw7−/− animals, despite such an increase having been reported previously22 23 and being present in our R482Q mutants. There was no good evidence that levels of other known FBXW7 substrates were increased in the mice studied, suggesting that their roles in intestinal tumorigenesis are limited. Current evidence does not suggest that the R482Q mutation affects a qualitatively different range of substrates from the null allele, since Tgif1, Klf5 and cyclin E were all significantly increased in Fbxw7−/− mice as well as in R482Q mutants. Evidently, it remains possible that unidentified FBXW7 substrates contribute to tumorigenesis and are differentially affected by propeller tip and protein truncating mutations.

Propeller tip mutant FBXW7 may act via a dominant negative mechanism

Existing evidence showed that propeller tip mutant FBXW7 can act as a dominant negative,12 and our own co-immunoprecipitation data supported this by showing that mutant and wild-type Fbxw7 proteins interact (figure 5A). Dominant negative loss of function is therefore the most likely mechanism underlying the more severe phenotype of R482Q+/+ mice. In further support of this model, we found that overexpression of wild-type FBXW7 causes TGIF1 levels to reduce significantly in colorectal cancer cell lines that carry propeller tip mutations (LOVO, heterozygous R505C and SW1463, heterozygous R479Q) (figure 5B). C32, a control cell line carrying a heterozygous protein truncating mutation of uncertain significance (R278X), showed no decrease in TGIF1 (figure 5B), suggesting that TGIF1 levels in these cells were lower than those in wild-type cell lines. The mechanism underlying the more severe phenotype of R482Q/+ animals, however, remains uncertain given the lack of evidence for a significant difference in the number (p=0.127) or size (p=0.661) of polyps between the different genotypes (figure 4). No significant differences were observed in the expression of other known FBXW7 substrates in the mice studied, suggesting that their roles in intestinal tumorigenesis are limited. Current evidence does not suggest that the R482Q mutation affects a qualitatively different range of substrates from the null allele, since Tgif1, Klf5 and cyclin E were all significantly increased in Fbxw7−/− mice as well as in R482Q mutants. Evidently, it remains possible that unidentified FBXW7 substrates contribute to tumorigenesis and are differentially affected by propeller tip and protein truncating mutations.

Figure 4 Kaplan–Meier plots of survival of R482Q/+ and Fbxw7−/− mice on an Apc mutant background. Note the more severe disease associated with the R482Q allele. Although not shown, survival of 1322T animals was consistently shorter than that of Min mice for both Fbxw7 alleles.

R482Q/+;1322T compared with 1322T tumours (p=0.045 and p=0.042, respectively, t test, figure 3), consonant with the findings in normal intestinal epithelium.

R482Q/+ is a stronger promoter of polyposis than Fbxw7−/− in Apc mutant mice

Our ‘just enough’ model of FBXW7 mutations predicts that R482Q heterozygotes have a greater functional effect than null heterozygotes. We therefore compared polyp formation in R482Q/+ and Fbxw7−/− mice on an Apc mutant (Min or 1322T) background using age of developing symptoms as a measure of disease severity. In an analysis stratified by type of Apc mutation, we found that R482Q/+ mice presented significantly earlier than Fbxw7−/− animals (mean 101 vs 122 days, p=0.001, Cox proportional hazards test, figure 4). No significant difference in the number (p=0.127) or size (p=0.661) of polyps between the different Fbxw7 models at presentation was found, consistent with tumour load being the principal factor determining age of onset of symptoms. The R482Q allele was, however, additionally associated with a shift in polyp distribution to SB1 (39% in R482Q/+ vs 26% in Fbxw7−/−, p=0.024). Analysis of only two Fbxw7−/− animals was possible, and their age of presentation was not significantly different from the R482Q/+ animals.
In summary, our data support the ‘just enough’ model in showing that the R482Q mutation has a stronger effect on Apc-driven intestinal tumorigenesis than a null heterozygote (Fbxw7+/-) mutation. Tgif1 and Klf5, rather than the classical Fbxw7 substrates, are the best candidate mediators of this differential effect, although the underlying mechanism requires further analysis. Contrary to the ‘just enough’ model, however, the murine Fbxw7+/− genotype did promote intestinal tumorigenesis, whether alone or on an Apc mutant background, whereas we predict that this genotype causes insufficient functional derangement to promote human tumorigenesis. This discrepancy may be a mouse-specific phenomenon resulting from a decreased requirement for ‘second hits’ at Fbxw7 compared with humans, as our in vitro data suggest; or it may reflect the fact

![Figure 5](image-url)

**Figure 5** In vitro studies of FBXW7 in colorectal cancer cell lines. (A) Interaction of mouse wild-type and R482Q Fbxw7 protein: co-immunoprecipitation (Co-IP) experiments of tagged Fbxw7 R482Q (FLAG) and wild-type (Myc) plasmids transiently transfected into RKO cells (FBXW7 wild-type). In FLAG Co-IP, lane 1, cells were transfected with both plasmids and FLAG co-immunoprecipitation performed; western blotting using anti-Myc identifies wild-type Fbxw7 (left, upper), indicating that R482Q mutant and wild-type Fbxw7 proteins interact. Anti-FLAG western blot control is shown (left, lower). In the reverse Co-IP using anti-Myc, western blotting using anti-FLAG similarly identified R482Q mutant Fbxw7 (right, upper). Anti-Myc western blot control is shown (right, lower). These experiments show that Fbxw7 wild-type and R482Q proteins interact. (B) Western blot of the nuclear fraction of a panel of cell lines expressing exogenous wild-type FBXW7 shows significant decreases in TGIF1 levels in propellor tip mutant cells, but not heterozygous null mutant or wild-type cells. The graph shows quantitation of the western blots by measuring average pixel intensities. Error bars represent SEM. *Significant results (LOVO p=0.006, SW1463 p=0.035, t test). Blots probed with anti-FLAG indicate transfection efficiency. KLF5 levels were too low in some of these cell lines for reliable analysis. (C) Knockdown of FBXW7 using short hairpin (shRNA) in the propellor tip mutant cell line LOVO causes no significant increase in TGIF1 protein levels (p=0.477, t test). Six and seven are results from different FBXW7 shRNA clones. The graph show the ratio of normalised pixel intensities relative to the cells transduced with scrambled shRNA as a control.
that heterozygous null human FBXW7 genotypes do have some, submaximal, tumour promoting effects, perhaps in a continuum fashion. Overall, our data support the notion that FBXW7 is not a classical tumour suppressor gene. As propellor tip mutations require only ‘one hit’ and yet have strong enough functional effects for tumorigenesis, they occur more often than the alternative of biallelic protein inactivating mutations.

Correction notice The license of this article has also changed since publication to CC BY 4.0.

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