T-cell acute lymphoblastic leukemias express a unique truncated FAT1 isoform that cooperates with NOTCH1 in leukemia development

The human *FAT1* gene was cloned over 20 years ago,[1] but there has only been an incremental understanding of its functional role in cancer and developmental disorders.[2] FAT1 is highly expressed in a large proportion of cases of T-cell acute lymphoblastic leukemia (T-ALL) and B-cell ALL compared to their normal counterparts suggesting an oncogenic function.[3] Conversely, the *FAT1* gene is also recurrently mutated in a small subset of T-ALL cases[4,5] and also in chronic lymphocytic leukemia.[6] Functionally, the FAT1 cadherin has been implicated in Wnt signaling,[7] hippo signaling[8] and more recently mitochondrial function[9] which together suggests a role outside the classical cadherin function in regulating cell-cell adhesion. Here we show that T-ALL cell lines and clinical samples express a unique N-terminal truncated *FAT1* mRNA transcript that generates a protein from a novel transcriptional start site within a retained intronic sequence. This novel transcript is regulated independently of full-length *FAT1* and results in the expression of a truncated protein lacking almost the entire extracellular domain of FAT1. Significantly, this truncated protein is a novel biomarker for T-ALL and was found to cooperate with NOTCH in driving T-ALL *in vivo*, suggesting that in the context of T-ALL, this truncated protein may act as an oncogene.

Examining the levels of *FAT1* expression in sorted normal healthy hematopoietic and T-cell progenitors revealed that *FAT1* is expressed at low levels in all normal T-cell subsets (Figure 1A). In contrast, *FAT1* is expressed at significantly higher levels in T-ALL cell lines (Figure 1B). Analysis of *FAT1* expression in different molecular-genetic subtypes of human T-ALL[10] revealed that *FAT1* expression is low or absent in immature T-ALL but expressed at higher levels in TAL1-positive T-ALL cases and variably expressed in TLX1-, TLX3- and HOXA-positive subsets of T-ALL (Figure 1C, *Online Supplementary Figure S1*).

In the original paper reporting the molecular cloning of the *FAT1* transcript, multiple smaller truncated *FAT1* transcripts were described in T-ALL cell lines but were not well characterized.[1] To investigate this further we performed Northern blotting with a 3'cDNA probe encoding nucleotides 13091-14751 of *FAT1* mRNA. This analysis revealed T-ALL cell lines uniquely express 5' truncated

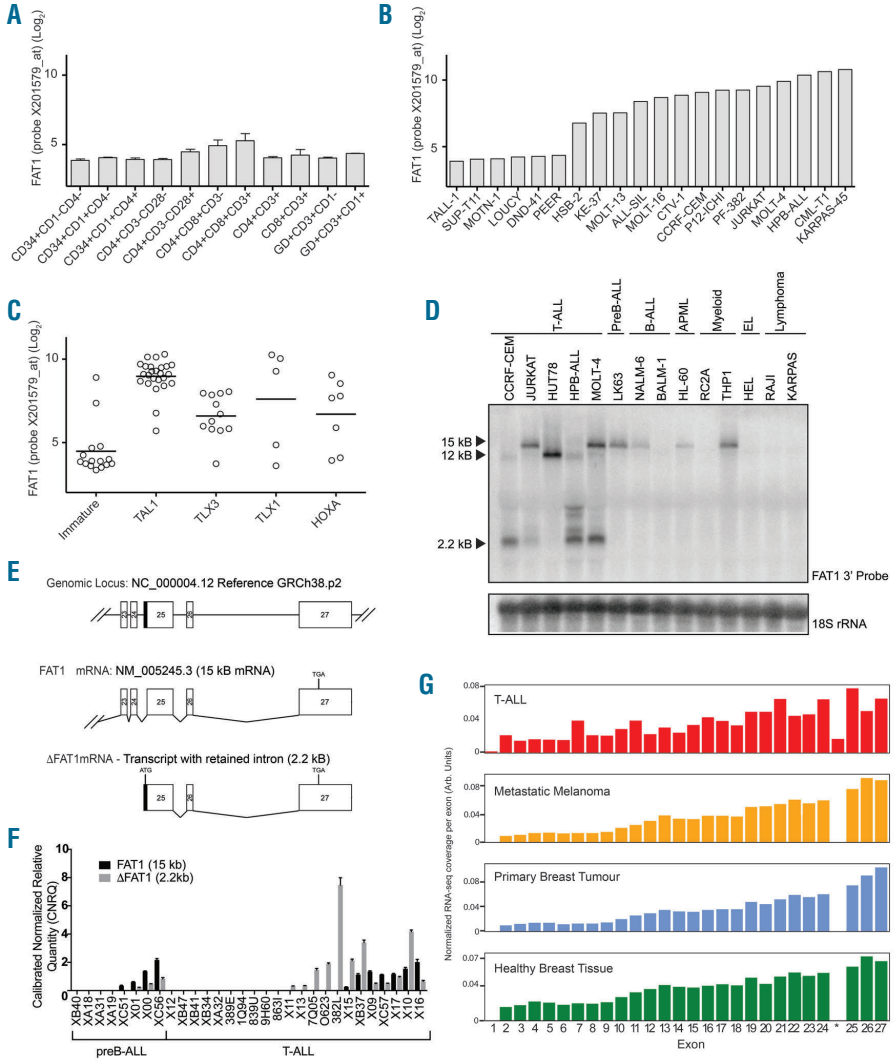

Figure 1. Truncated *FAT1* transcript with a retained intron is uniquely expressed in T-ALL. (A) *FAT1* is expressed at low levels across a diverse range of normal T-cell subsets. (B) In contrast 14/20 T-ALL cell lines expressed high levels of *FAT1* transcript. (C) In clinical T-ALL cases, *FAT1* is expressed predominantly within TAL1-positive cases and is negatively correlated with immature cases of T-ALL. (D) Northern blot analysis of T-ALL cell lines identified multiple 5' truncated *FAT1* transcripts present with the majority expressing a band migrating at 2.2 kb. (E) Schematic representation of the 3' exon structure of the *FAT1* gene locus and the 5' RACE result showing that the 2.2 kb truncated transcript contains a retained intronic sequence directly upstream of exon 25. (F) Real-time polymerase chain reaction analysis of B- and T-ALL patient-derived xenograft samples shows that full-length *FAT1* is expressed in both T-ALL and B-ALL cases but the Δ*FAT1* transcript is present at high levels only in T-ALL cases. (G) Analysis of the TCGA and TARGET databases revealed that the retained intronic sequence denoted by * is unique to T-ALL cases when compared to melanoma and breast cancers that also have high levels of *FAT1* expression.

transcripts of *FAT1*. These predominantly comprised a high molecular variant migrating at approximately 12 kB in the HuT78 cell line and two smaller variants of approximately 2.2 kB and 2.5 kB in CCRF-CEM, Jurkat, MOLT-4 and HPB-ALL cell lines (Figure 1D). Exon-specific Northern blotting and reverse transcriptase polymerase chain reaction (PCR) showed that the larger 12 kB transcript in HuT78 lacked exons 1 and 2 with no corresponding genomic deletion, while the 2.5 kb transcript lacked exons 1-22 and the 2.2 kb fragment lacked exons 1-24 (*Online Supplementary Figure S2A-E*).

The smaller 2.2 kb and 2.5 kb transcripts were charac-

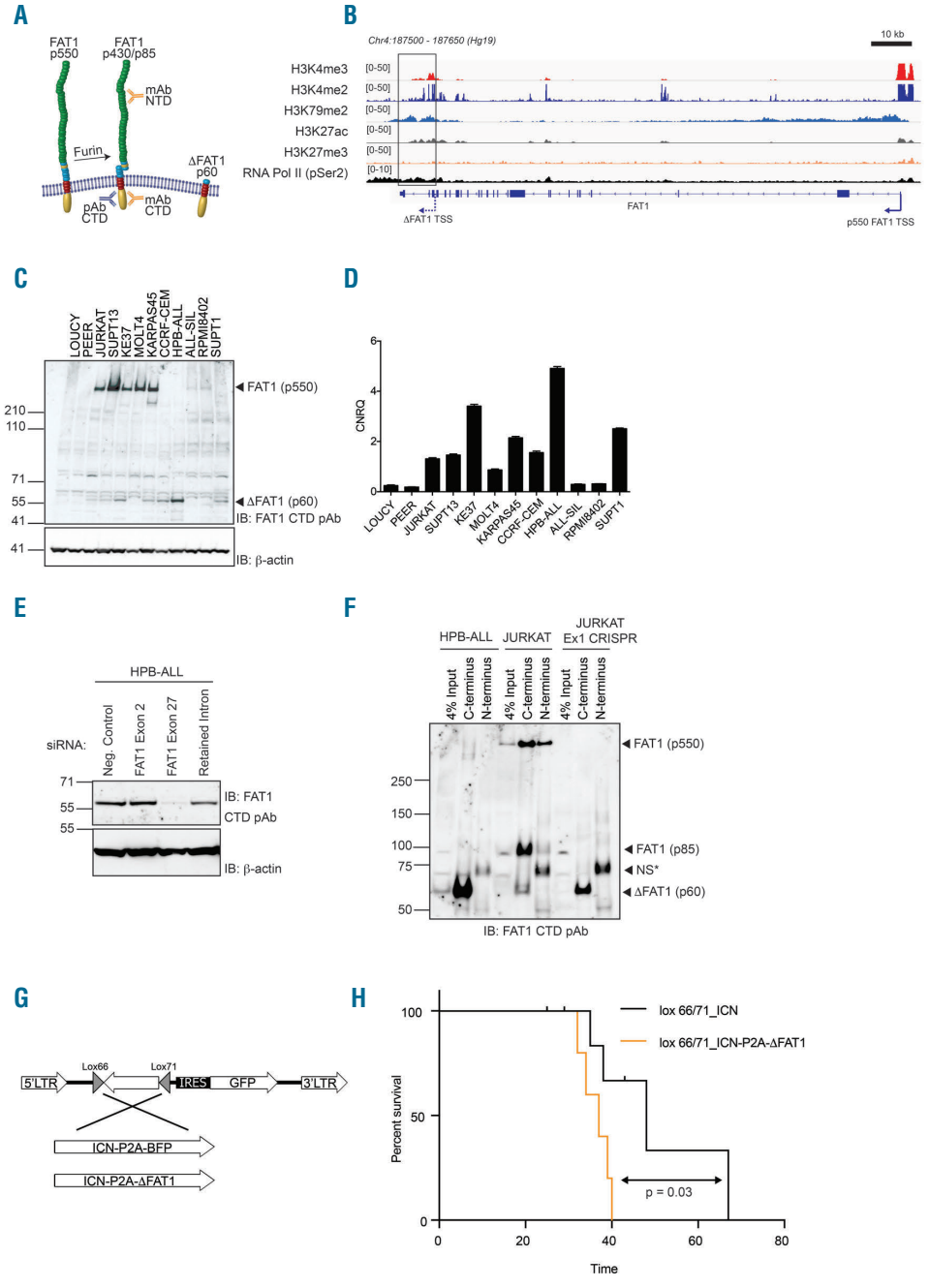

Figure 2. The truncated *ΔFAT1* transcript produces a protein independent of full-length FAT1. (A) Schematic showing the full-length FAT1 (p550) protein which undergoes furin-mediated cleavage to produce a (p450/p85) heterodimer alongside the predicted truncated ΔFAT protein. (B) Chromatin immunoprecipitation-sequencing data for histone marks and RNA-pol II (pSer2 C-terminal domain) across the *FAT1* genetic locus in the Jurkat cell line. The transcriptional start sites (TSS) for *FAT1* and *ΔFAT1* are highlighted. (C) Western blot analysis of a panel of T-ALL cell lines using an antibody directed against the cytoplasmic tail of FAT1 identified both full-length p550 FAT1 and a specific band migrating at the predicted molecular weight for ΔFAT (p60). (D) Real-time polymerase chain reaction analysis of the levels of *ΔFAT1* mRNA transcript reconciles directly with the Western blot analysis. (E) HPB-ALL cells only express the ΔFAT1 protein and siRNA directed toward exon 27 (the cytoplasmic tail) ablated expression while siRNA directed toward exon 2 of *FAT1* was equivalent to a negative control. A siRNA directed toward the small intronic sequence also reduced the expression of ΔFAT1. (F) CRISPR/Cas9-mediated knockout of *FAT1* using a guide RNA directed toward exon 1 completely removed full-length FAT1 (p550) protein expression but did not reduce ΔFAT1 p60 expression in Jurkat cells. HPB-ALL cells exclusively expressed ΔFAT which was only detected after immunoprecipitation using C-terminal but not N-terminal directed antibodies (*NS = nonspecific band). (G) Schematic representation of the inducible retroviral constructs used to express either ICN or ICN and ΔFAT1 in the presence of Cre-recombinase. (H) Bone marrow transplant survival curve for CD2-Cre-driven expression of either ICN only or ICN and ΔFAT1 in early T-cell progenitors (log-rank, Mantel-Cox test, *P*=0.03).

terized using 5' RACE from total RNA isolated from Jurkat cells. Two major transcript clones were sequenced and found to include retained intronic sequences at their 5' ends (*Online Supplementary Table S1*). The shorter 2.2 kb transcript had a retained intronic sequence mapping directly upstream of exon 25 (Figure 1E). This transcript is homologous with a previously annotated Ensembl FAT1-004 transcript (OTTHUMT00000360219) and partially aligns with the EST transcript BX362336.2 deposited in Genbank.

The clinical relevance and expression of the FAT1-004 transcript (ΔFAT1 for the remainder of this report) was defined in a panel of primary T-ALL and B-ALL samples (*Online Supplementary Table S2*) using primer-specific real-time PCR to quantify both full-length *FAT1* and Δ*FAT1* mRNA levels. This analysis found that 32% (7/22) of the T-ALL samples expressed full-length *FAT1* but, significantly, 55% (12/22) expressed the Δ*FAT1* mRNA transcript. In five T-ALL cases, only the Δ*FAT1* mRNA transcript was present with no full-length *FAT1* mRNA. Of the eight preB-ALL cases, four (50%) had full-length *FAT1*, and three of these cases also had detectable Δ*FAT1* transcript albeit always at substantially lower levels compared to those in T-ALL and this was speculated to reflect low levels of pre-mRNA (Figure 1F). Analysis of RNA-sequencing data deposited in the TCGA and TARGET databases found that reads covering the Δ*FAT1* retained intronic sequence upstream of exon 25 (indicated by *) were unique to T-ALL and not present in melanoma and breast cancer, previously described as having high levels of *FAT1* expression[11,12] (Figure 1G).

In silico analysis of the retained intronic sequence of the Δ*FAT1* transcript revealed the presence of an upstream in-frame ATG (methionine) start codon suggesting that the Δ*FAT1* transcript has the potential to generate a protein. This protein would consist of a single extracellular EGF-like domain, a transmembrane region and the entire cytoplasmic tail (Figure 2A), and is predicted to be expressed on the plasma membrane (*Online Supplementary Figure S3*). The independent regulation of Δ*FAT1* is supported by chromatin immunoprecipitation-sequencing data from Jurkat cells that have a strong promotor H3K4me3 signal surrounding the putative start site of the Δ*FAT1* transcript together with strong H3K27ac and H3K79me2 marks indicating open chromatin and a transcriptional transition region, respectively, and strong RNA pol II loading (Figure 2B). Moreover, the H3K27Ac mark at the Δ*FAT* region was consistent among T-ALL cells lines that express the Δ*FAT1* transcript and absent from those cell lines that do not express FAT1 (e.g. LOUCY) (Figure 2B) or other cells lines known to express only full-length FAT1 (e.g. MDA-MB-231 and 293T) (*Online Supplementary Figure S4*).

Expression of the truncated protein was confirmed by Western blotting of a panel of T-ALL cell lines using a rabbit polyclonal antibody raised against the cytoplasmic tail of *FAT1* which revealed a prominent immunoreactive band of the predicted size for Δ*FAT1* at ~60 kDa in eight of 12 T-ALL lines (Figure 2C). Protein expression corresponded with levels of Δ*FAT1* mRNA (Figure 2C,D). As determined by Northern blotting, HPB-ALL cells exclusively expressed the Δ*FAT1* transcript (Figure 1D) and had a strong immunoreactive band at ~60 kDa that was lost through specific short interfering (si)RNA targeting of FAT1 exon 27 but not siRNA targeting of *FAT1* exon 2 (Figure 2E). A custom-designed siRNA, albeit with low efficacy parameters due to the short non-optimal intronic sequence, also resulted in reduced expression of the ΔFAT1 protein (Figure 2E).

In solid tumors, FAT1 undergoes furin-mediated post-translational cleavage to generate a p430/p85 heterodimer on the cell surface as well as ADAM10-mediated ectodomain shedding.[11] To confirm that the ΔFAT1 protein is neither a splice variant of full-length *FAT1* mRNA nor the result of any post-translational cleavage events, immunoprecipitation was carried out using FAT1 antibodies targeting either the extracellular domain or intracellular cytoplasmic domain on HPB-ALL, Jurkat and a CRISPR/Cas9 Jurkat cell line targeting exon 1 of *FAT1* to selectively target only the high molecular weight FAT1 (Figure 2F). HPB-ALL cells had a strong enrichment for ΔFAT1 only with the antibody directed toward the cytoplasmic domain and not with the N-terminal targeting anti-FAT1 antibody. In Jurkat cells, there was enrichment for the full length FAT1 with both N-terminal and C-terminal antibodies and ΔFAT1 was only present when immunoprecipitated with the C-terminal domain-directed antibody. When full-length FAT1 was removed by CRISPR/Cas9 guide RNA directed toward *FAT1* exon 1 in Jurkat cells, there was no full-length FAT1 present confirming successful loss of FAT1 protein. Significantly, the expression of the novel ΔFAT1 protein remained after immunoprecipitation with the FAT1 C-terminal domain antibody.

The ΔFAT1 truncated protein product was strikingly reminiscent of the *Gull* mutant allele occurring in *Drosophila fat* that leads to overgrowth of the wing imaginal disc.[13] Similarly, overexpression of ΔFAT1 led to increased cell proliferation and colony formation according to *in vitro* murine cell-based transformation assays (*Online Supplementary Figure S5*). However, in an *in vivo* bone marrow transplant model, ΔFAT1 expression only led to modest B-cell expansion and no overt leukemia within the 200-day observation period (*Online Supplementary Figure S6*). In the context of T-ALL, chromatin immunoprecipitation-sequencing for activated Notch1 (ICN) shows a specific binding peak upstream of the putative ΔFAT1 transcriptional start site[14] suggesting that ICN and ΔFAT1 may cooperate in the development and/or progression of T-ALL. However, *in vitro* siRNA knockdown of NOTCH1 did not alter levels of Δ*FAT1* mRNA after 24 h suggesting that other factors must also regulate Δ*FAT1* expression (*Online Supplementary Figure S7*). However, loss of both NOTCH1 and ΔFAT1 did lead to a decrease in MYC expression in ALL-SIL and CCRF-CEM (*Online Supplementary Figure S7*). Therefore, to determine the leukemogenic potential of ΔFAT1, a bone marrow transplant model was used to express ΔFAT1 in an inducible manner with ICN using a novel Cre-inducible retroviral construct in conjunction with a CD2-Cre mouse.[15] When ΔFAT1 was expressed with ICN using the inducible CD2-Cre model, mice rapidly developed the expected $CD4^+/CD8^+$ T-ALL (*Online Supplementary Figure S8*) with the combination leading to a significant decrease in leukemic latency *in vivo* revealing that this truncated FAT1 protein acts in an oncogenic manner in the context of NOTCH1-induced T-ALL (Figure 2G,H).

In conclusion, these data show that T-ALL express a unique and novel truncated FAT1 protein that is not only a specific biomarker for T-ALL, but also cooperates with mutant NOTCH1 in driving the disease. This finding urges caution in defining and interpreting the functional role of full-length FAT1 when using recombinant FAT1 constructs that delete the FAT1 extracellular domain[7] as these may potentially act as dominant negative for full-length FAT1 signaling, or have *de novo* protein signaling functions.

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