

# BTK-T474I with enhanced kinase activity confers growth advantage over BTK-L528W with kinase deficiency in B malignant cells



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#abstract No. 5600

## BACKGROUND

Covalent BTKi (cBTKi) have transformed the therapeutic landscape for chronic lymphocytic leukemia (CLL)<sup>1,2</sup>. However, acquired mutations in BTK, often occurring at C481, lead to drug resistance and disease progression<sup>3</sup>. While non-covalent BTKi (ncBTKi) has demonstrated promising efficacy in CLL patients with BTK C481X mediated resistance, non-C481 mutations within BTK kinase domain confer ineffectiveness to cBTKi and ncBTKi as well<sup>4,5</sup>. Those BTK mutations most commonly involve T474I and L528W<sup>5,6</sup>. To better understand their role in supporting B malignant cell growth, we developed clonal competition models by co-culturing isogenic B malignant cells harboring BTK-T474I and BTK-L528W and monitor the clonal dynamics in long term culture. Additionally, we conducted mechanistic analysis utilizing proteomics and phospho-proteomics.

## METHODS

T474I and L528W were introduced to BTK allele of B malignant cell line TMD8. BTK downstream signaling in BTK wildtype or mutant cells with or without anti-IgM stimulation was assessed by western blot assay. *In vitro* and *in vivo* models containing TMD8 wildtype and mutant cells were employed to investigate the clonal competition. The dynamics of individual clone were monitored by measuring BTK variant allele frequency (VAF) via amplicon sequencing. TMD8 cells expressing wildtype BTK, BTK-T474I (ENU clone), or BTK-L528W (KI #1) were cultured in medium with 2% FBS for 14 days. Cells were treated with 10 µg/ml anti-IgM or not. Samples were subjected to total proteomics and phospho-proteomics analysis.

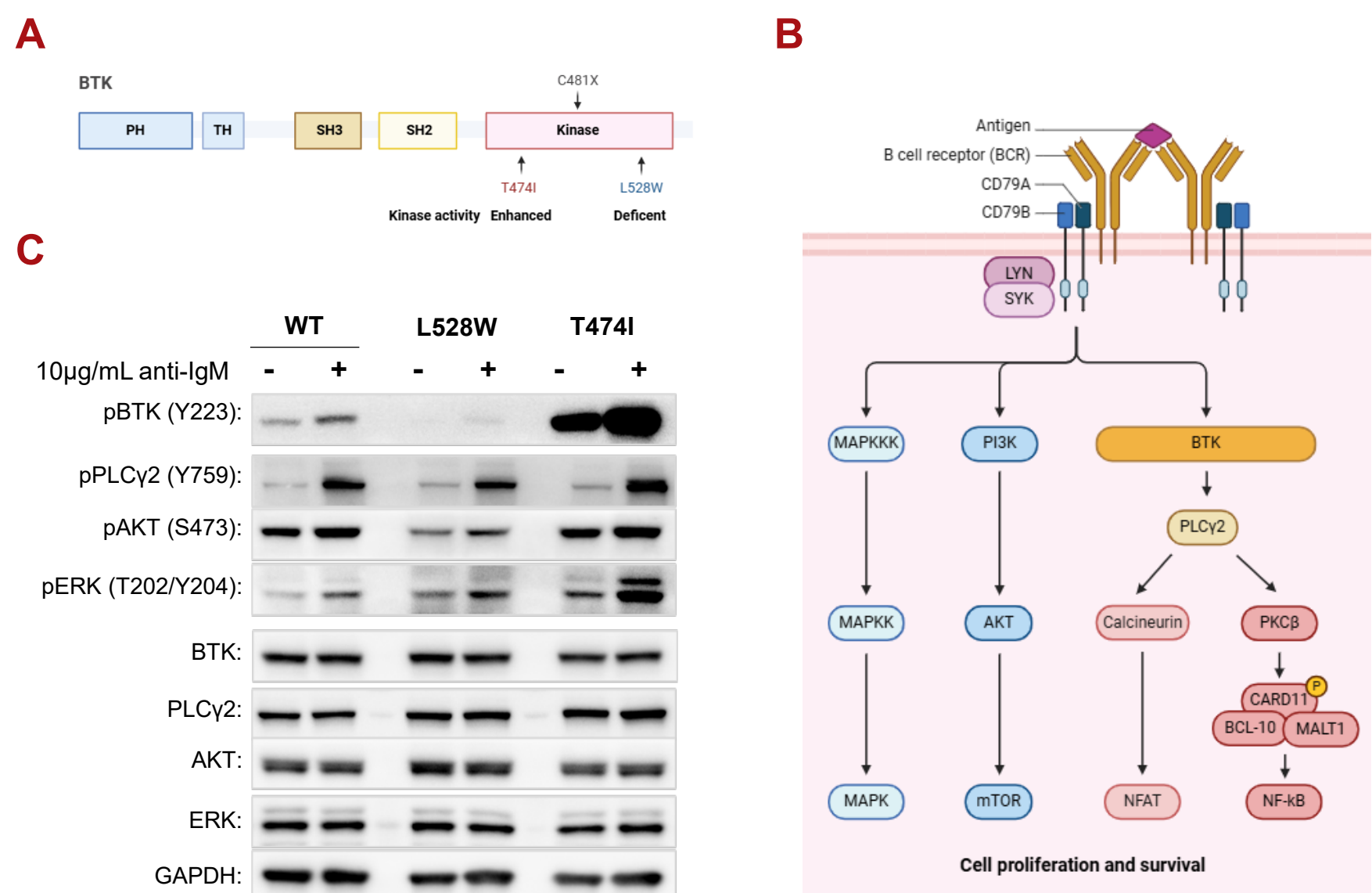
## RESULTS

Hyperactivation of phosphorylated BTK Y223 in TMD8 BTK-T474I cell and minimal BTK Y223 phosphorylation in TMD8 BTK-L528W upon IgM stimulation was observed. Enhanced BTK kinase activity indicated by downstream signaling (pPLCγ2, pERK and pAKT) were noted in TMD8 BTK-T474I cells compared to BTK-L528W cells. Those findings suggested that BTK-T474I encodes a kinase with enhanced activity while BTK-L528W results in a catalytically inactive form. Clonal competition was evaluated using co-culture models containing TMD8 BTK-T474I, BTK-L528W and wildtype cells in medium with various FBS concentration. BTK T474I clones were significantly expanded while BTK L528W clones were declined in 4 weeks culture period. An increase in BTK T474I VAF and a decrease in BTK L528W VAF were observed during tumor growth in subcutaneous models incorporating TMD8 wildtype and mutant cells. Total proteomics analysis revealed an enhanced cell cycle progression in BTK-T474I cells compared to BTK-L528W cells, as indicated by upregulation of cell cycle-related proteins. In addition, increased kinase activity of key components within BCR pathway was demonstrated through phospho-proteomics.

## CONCLUSIONS

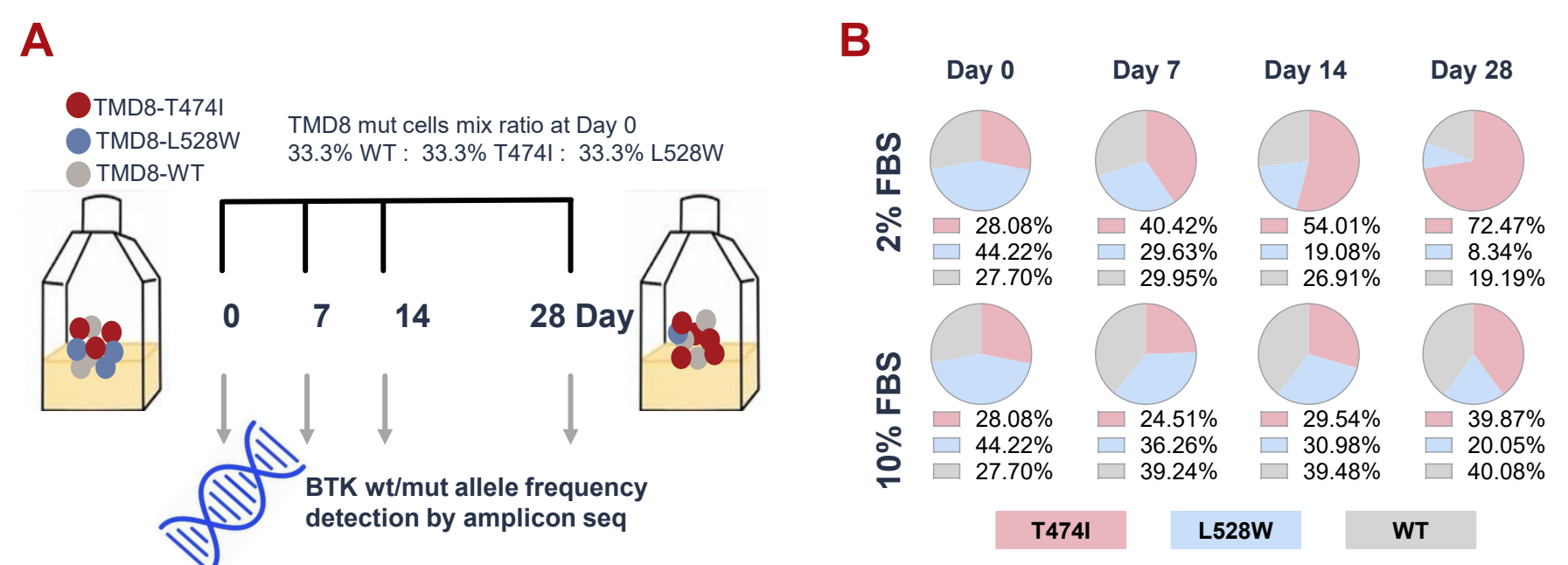
Our *in vitro* and *in vivo* clonal competition assays provided novel insights into the impact of BTK-T474I and BTK-L528W on B malignant cell growth fitness. These findings suggested that BTK-T474I, characterized by enhanced kinase activity, confers growth advantage over the kinase deficient form BTK-L528W, and provided evidence of alternative signaling mechanism between BTK mutants with enhanced or impaired kinase activity.

**Figure 1. Enhanced BTK kinase activity in TMD8 BTK-T474I than TMD8 BTK-L528W cells**



(A) Diagram of covalent or non-covalent BTK inhibitor resistant mutation C481X, T474I, L528W in BTK kinase domain. (B) Schematic representation of BCR/BTK signaling pathway. (C) TMD8 cells expressing BTK wildtype (WT), T474I or L528W were incubated with 10µg/ml anti-IgM for BCR signaling stimulation. Phosphorylation of BTK Y223, PLCγ2 Y759, AKT S473, and ERK T202/Y204 were examined by western blot assay. Hyperactivation of BTK Y223 in TMD8 BTK-T474I cell whereas minimal BTK Y223 phosphorylation in TMD8 BTK-L528W cells were noted. Enhanced BTK kinase activity, as indicated by pPLCγ2, pERK, and pAKT levels, was observed in TMD8 BTK-T474I cells compared to TMD8 BTK-L528W cells.

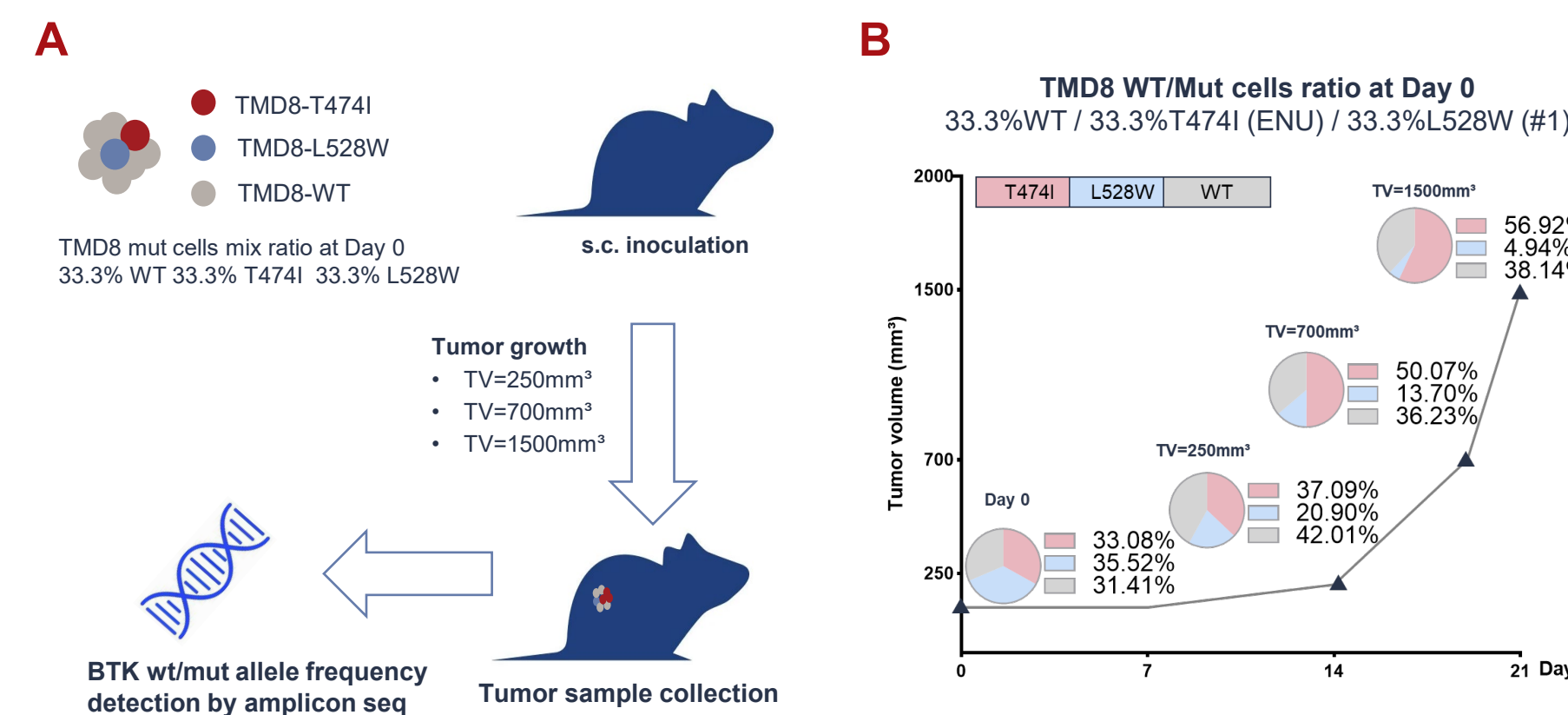
**Figure 2. TMD8 BTK-T474I cells derived from ENU mutagenesis show higher expansion potential over TMD8 BTK-L528W cells in *in vitro* clonal competition assay**



(A) Workflow of *in vitro* clonal competition assay. TMD8 wildtype cells, TMD8 BTK-T474I cells derived from ENU (N-ethyl-N-nitrosourea) mutagenesis or TMD8 BTK-L528W cells (knock-in clone #1) were mixed in equal proportion on day 0 and cultured in medium with 2% or 10% of FBS. Cell pellets were collected at specified time points for amplicon sequencing to determine the allele frequency of BTK wildtype or mutants. Results are illustrated in (B). An increase in T474I variant allele frequency (VAF) and a decrease in L528W VAF were observed following 4 weeks of cell culture. The growth advantage of TMD8 BTK-T474I over TMD8 BTK-L528W was particularly pronounced when cultured in medium with lower FBS concentration, which simulates relatively harsh condition for cell proliferation.

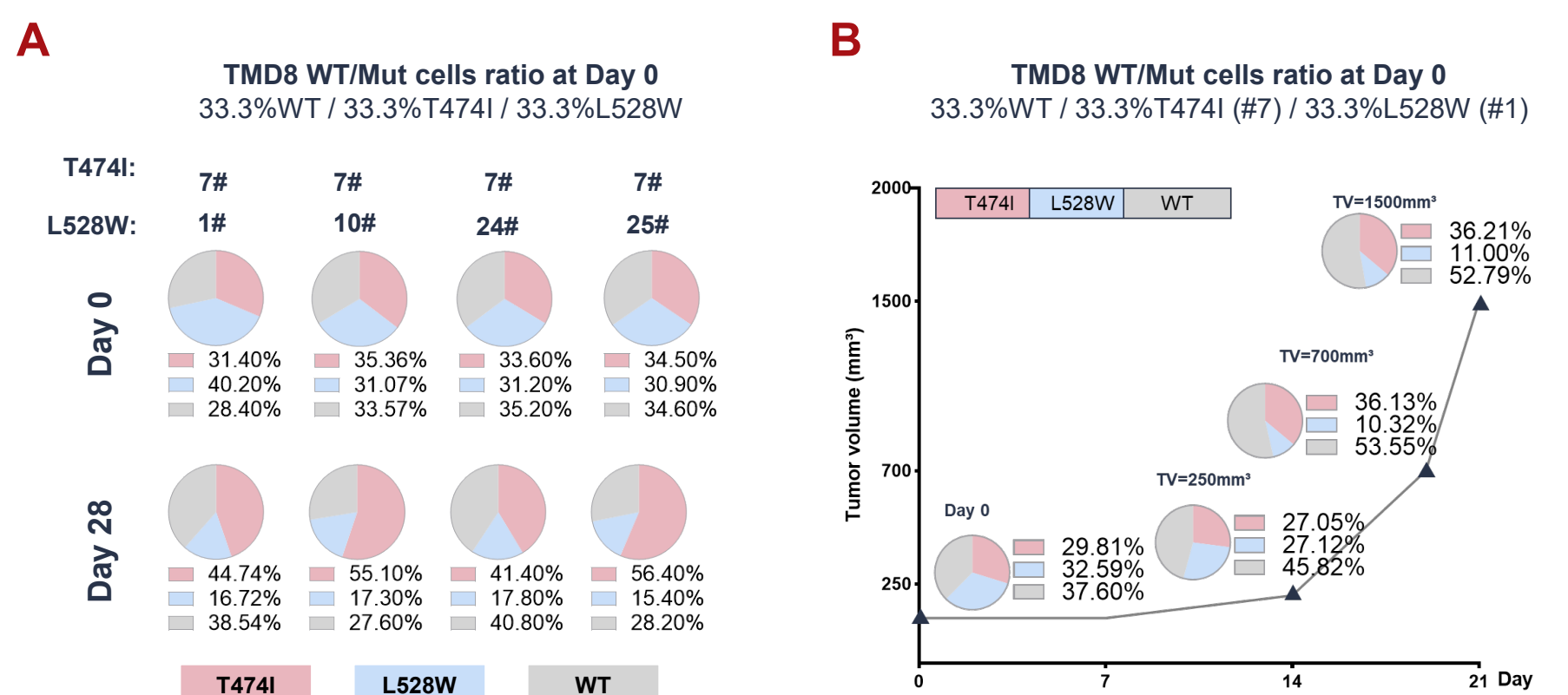
Reference: 1. N Engl J Med 2013;369:32-42; 2. N Engl J Med. 2023 Jan 26;388(4):319-332; 3. N Engl J Med. 2014 Jun 12;370(24):2286-94; 4. N Engl J Med. 2023 Jul 6;389(1):33-44; 5. N Engl J Med. 2022 Feb 24;386(8):735-743;6. Blood (2023) 142 (Supplement 1): 326.

**Figure 3. TMD8 BTK-T474I cells derived from ENU mutagenesis exhibit outgrowth advantage over TMD8 BTK-L528W cells in *in vivo* clonal competition assay**



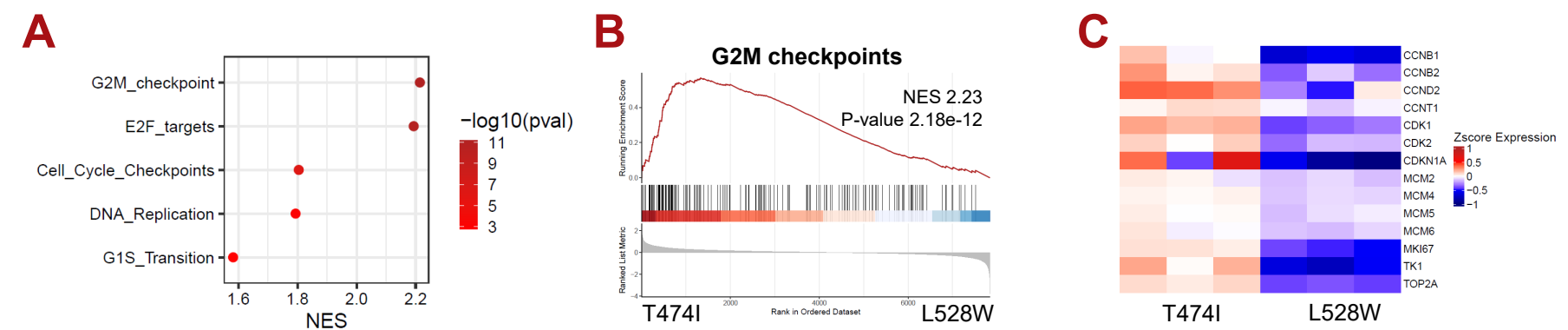
(A) Workflow of *in vivo* clonal competition assay. Cells with wildtype BTK, BTK-T474I and BTK-L528W were mixed in equal proportion and injected into mice on day 0 subcutaneously. Cell mixture pellets from day 0 and tumors at average sizes of 250 mm<sup>3</sup>, 700 mm<sup>3</sup>, and 1500 mm<sup>3</sup> (N=3) were collected for amplicon sequencing to determine the allele frequencies of BTK wildtype and mutants. The dynamic changes of clone composition for tumors over time were depicted in (B). A significant increase in TMD8 BTK-T474I cells and a corresponding decrease in TMD8 BTK-L528W cells were observed.

**Figure 4. BTK-T474I conferring *in vitro* and *in vivo* growth advantage over BTK-L528W was confirmed by independent TMD8 BTK-T474I and BTK-L528W clones**



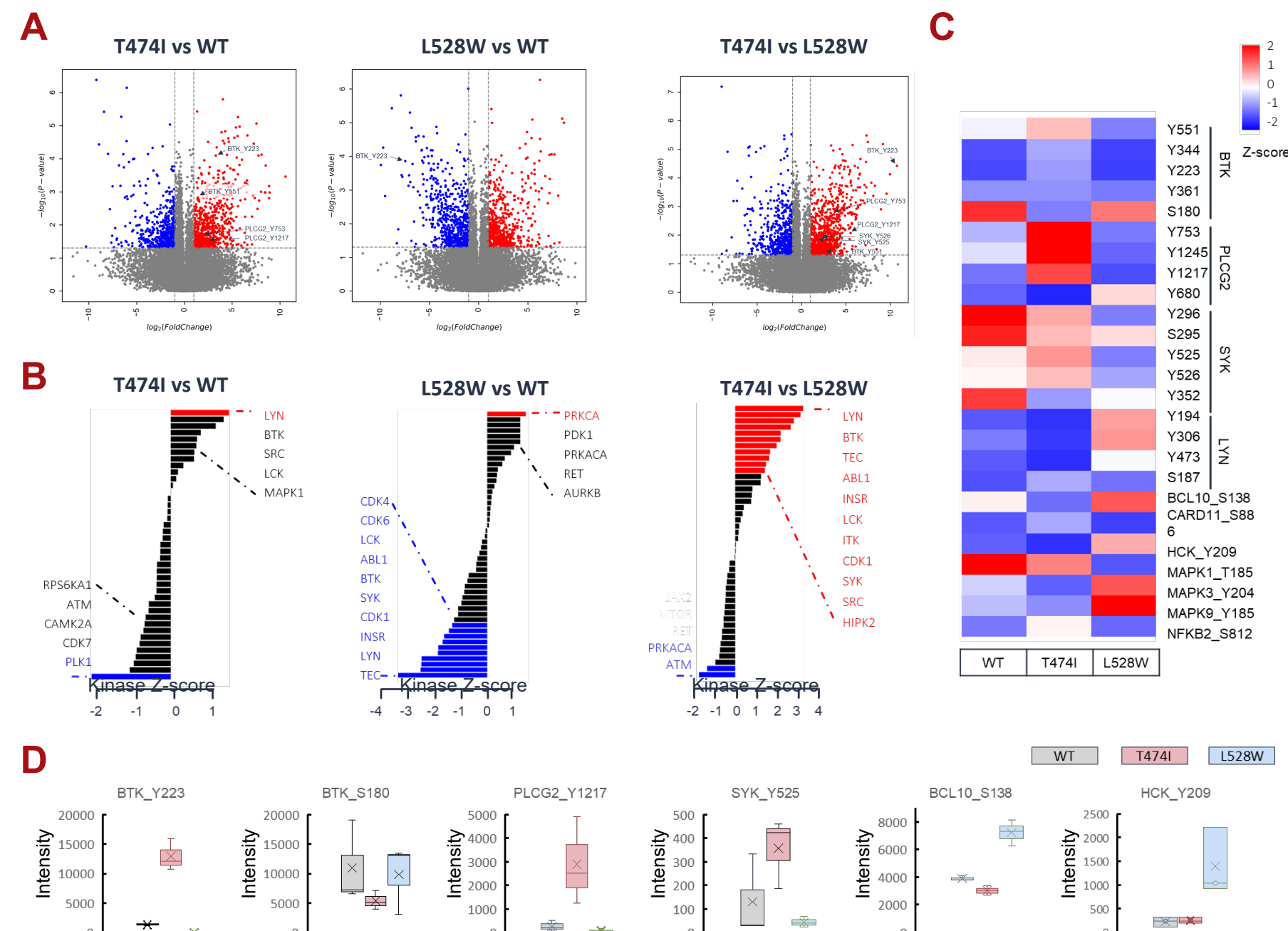
The growth advantage of cells harboring BTK-T474I compared to those with BTK-L528W was confirmed through *in vitro* and *in vivo* clonal competition assays, using an independent BTK-T474I knock-in clone (#7) and multiple BTK-L528W knock-in clones. (A) TMD8 cells expressing wildtype BTK, BTK-T474I (KI #7), and various BTK-L528W clones (KI #1, #2, #10, #25) were mixed in equal proportion on day 0 and cultured in medium supplemented with 2% FBS. Cell pellets were collected on day 28 for amplicon sequencing to assess the allele frequency of BTK variants. An increase in variant allele frequency (VAF) of BTK-T474I and a corresponding decrease in VAF for BTK-L528W were observed across four pairwise comparisons. (B) Enhanced outgrowth potential of TMD8 cells carrying BTK-T474I (KI #7) relative to those with BTK-L528W (KI #1) was demonstrated in *in vivo* clonal competition assay.

**Figure 5. Enhanced cell cycle progression in TMD8 BTK-T474I than TMD8 BTK-L528W cells revealed through proteomics analysis**



TMD8 cells expressing BTK-T474I (ENU clone), BTK-L528W (KI #1) were cultured in 2% of FBS for 14 days and subjected to proteomics analysis. GSEA top up-regulated pathways in TMD8 BTK-T474I vs BTK-L528W cells (A) and GSEA of G2M checkpoints (B) showed that the differentially expressed proteins were mainly enriched in cell cycle related pathways. (C) Heatmap illustrated the expression level of cell cycle associated proteins in TMD8 BTK-T474I and BTK-L528W cells.

**Figure 6. TMD8-BKT T474I showed stronger BCR signal than BTK-L528W cells by phospho-proteomics analysis**



TMD8 cells with BTK WT, BTK-T474I (ENU clone), BTK-L528W (KI #1) were treated with 10 µg/ml anti-IgM and then subjected to phospho-proteomics analysis. (A) Volcano plots showed differentially phosphorylation sites between groups. Fold change of phosphorylation level for BTK, PLCγ2, SYK are labelled. (B) Kinase substrate enrichment analysis predicted kinase activity difference between groups by calculating hypo/hyper phosphorylated substrates. Significantly active (red) and inactive (blue) kinase between groups are highlighted. (C) Heatmap illustrated the phosphorylation status of key components of BCR pathway and its downstream effectors at indicated residues upon anti-IgM stimulation among groups. (D) Phosphorylation levels of proteins in BCR signalosome among groups. Elevated kinase activity for proteins within BCR pathway, including BTK, SYK, and PLCγ2, was observed in TMD8 BTK-T474I cells compared to BTK-L528W cells. Upregulation of phosphorylation sites indicative of enhanced kinase activity, along with decrease in phosphorylation sites that negatively regulate kinase activity (BTK S180), was evident in TMD8 BTK-T474I relative to BTK-L528W cells.