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Organelle proteomics reveals novel metabolic vulnerabilities in FLT3-ITD cells

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In acute myeloid leukemia (AML), the insertion site of internal tandem duplications (ITDs) within the *FLT3* gene critically determines the sensitivity to tyrosine kinase inhibitors (TKIs). Despite recent advances, patients harboring ITDs in the tyrosine kinase domain (TKD) still lack effective therapeutic options. To elucidate the molecular basis underlying the differential TKI sensitivity of FLT3-ITD cells, we integrated high-resolution mass spectrometry-based (phospho)proteomics with subcellular fractionation. Our analysis revealed that midostaurin induces the subcellular redistribution of approximately 2500 proteins involved in crucial biological processes, including cell cycle control, autophagy, and metabolism. Functional analyses further demonstrated that the ITD insertion site determines the autophagy response to midostaurin and modulates mitochondrial metabolism, influencing organelle architecture and ATP production, even at steady state. Importantly, by integrating subcellular proteomic dataset with functional metabolic assays, we uncovered a lipid-dependent vulnerability of FLT3-ITD cells: lipid restriction enhances FLT3 trafficking to the plasma membrane, and markedly reduces cell viability, restoring midostaurin sensitivity of resistant FLT3-ITD cells. Together, our findings reveal that the FLT3-ITD insertion site orchestrates a coordinated remodeling of subcellular protein organization, autophagy, and metabolism, and identify lipid-mediated control of FLT3 compartmentalization as a therapeutically actionable mechanism to overcome TKI resistance in FLT3-ITD AML.

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INTRODUCTION

Internal tandem duplications (ITDs) in the *FLT3* gene are detected in approximately 25–30% of younger adults with newly diagnosed acute myeloid leukemia (AML) and are associated with an adverse prognosis [1]. These mutations most commonly involve exons 14–15, affecting the juxtamembrane domain (JMD) or the first tyrosine kinase domain (TKD1), and resulting in constitutive FLT3 activation through disruption of its autoinhibitory conformation [2]. The implementation of the multikinase inhibitor midostaurin to standard induction and consolidation chemotherapy has improved outcomes for patients with *FLT3*-mutated AML [3, 4]. However, an ever-growing number of clinical and experimental evidence indicates that the prognostic and therapeutic impact of *FLT3*-ITD is influenced by the ITD insertion site [5]. While patients harboring JMD insertions (*FLT3*^{ITD-JMD}) benefit from FLT3 inhibition, ITDs within the TKD region (*FLT3*^{ITD-TKD}) are associated with reduced sensitivity to both chemotherapy and FLT3 tyrosine kinase inhibitors (TKIs), as well as lower relapse-free and overall survival. Consistent with these observations, experimental models demonstrate that *FLT3*^{ITD-TKD} mutations confer resistance to apoptosis in response to multiple TKIs, including gilteritinib and quizartinib, and to cytarabine, compared with *FLT3*^{ITD-JMD} cells

[6–9]. We recently demonstrated that the impact of ITD insertion site on TKI response is mediated by widespread phosphorylation changes, occurring independently of alterations in mRNA expression or protein abundance [6]. Based on these findings, we hypothesized that differential sensitivity of *FLT3*^{ITD-JMD} and *FLT3*^{ITD-TKD} cells to midostaurin is driven by phosphorylation-dependent redistribution of the proteome across subcellular compartments. To test this, we performed high-resolution mass spectrometry (MS)-based proteomics combined with subcellular fractionation. This approach identified approximately 1800 proteins exhibiting genotype-dependent changes in localization following midostaurin treatment. Notably, protein complexes involved in cell-cycle progression, DNA replication, and mitosis translocated from the nucleus to the cytosol exclusively in *FLT3*^{ITD-JMD} cells, underscoring the profound impact of ITD location on TKI-mediated cell-cycle control. Integration with phosphoproteomic data further revealed ITD-dependent differences in protein phosphorylation and trafficking, highlighting a different regulation of autophagic processes and distinct metabolic shifts in response to midostaurin. Specifically, we found that midostaurin induced autophagy and reduced oxidative phosphorylation and glycolysis in *FLT3*^{ITD-JMD} but not in *FLT3*^{ITD-TKD} cells. Furthermore,

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pharmacological inhibition of key metabolic pathways revealed that midostaurin-treated FLT3^{ITD-TKD} cells retain a partial dependence on lipid metabolism. Notably, lipid deprivation sensitized FLT3^{ITD-TKD} cells to TKI treatment, uncovering a novel metabolic vulnerability in FLT3-ITD AML cells. Overall, our work highlights the importance of integrating proteomic, phosphoproteomic, and metabolic analyses to uncover context-specific vulnerabilities in AML and offers a rationale for developing strategies aimed at overcoming resistance in high-risk FLT3-ITD patients.

MATERIALS AND METHODS

Cell culture

Mouse Ba/F3 cells expressing ITD-JMD and ITD-TKD constructs were provided by courtesy of T. Fischer. The cells were cultured in RPMI 1640 medium (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (ECS0090D Euroclone, Italy, MI), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco 15140122), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, Missouri, United States, S8636) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma H0887). Midostaurin (Selleck chemical, S8064) was used at 100 nM for 24 h.

RESULTS

The experimental strategy

To investigate how the FLT3-ITD insertion site shapes midostaurin-dependent proteome reorganization, we applied a streamlined spatial proteomics workflow [10] combining chemical fractionation with MS-based proteomics (Fig. 1A). This approach enables high-resolution mapping of protein relocalization across subcellular compartments and provides a quantitative, system-wide view of spatial changes in response to stimuli. Ba/F3 cells expressing FLT3^{ITD-JMD} and FLT3^{ITD-TKD} receptors were treated with 100 nM midostaurin (PKC412) for 24 h or left untreated. Sequential extraction using six buffers generated six subcellular fractions, each analyzed in biological quadruplicates. Label-free quantification yielded a comprehensive dataset of 8334 proteins (Fig. S1A, B), with ~5000 proteins consistently quantified across fractions (Pearson correlation 0.94–0.99; Fig. S1C–G; Fig. S2A; Table S1). As subcellular localization is dynamically regulated by post-translational modifications, we additionally profiled the global proteome and phosphoproteome in parallel (Fig. 1A, Table S2, S3). This analysis quantified marker proteins displayed precise distribution patterns across fractions as expected for this established protocol [10]: cytosolic markers were enriched in fraction 1; nuclear proteins in fractions 2 and 6; and lysosomal, Golgi, ER, mitochondrial, and endosomal proteins peaked in fractions 3–4 (Fig. 1B). Consistent with previous reports indicating perinuclear retention of FLT3-ITD mutants [11, 12], both FLT3^{ITD-JMD} and FLT3^{ITD-TKD} receptors showed maximal signal in fractions 3–4, corresponding to membrane-bound organelles (Fig. S2B). Clustering of protein abundance profiles across fractions revealed five major clusters corresponding to discrete subcellular compartments, with high concordance across genotypes and treatment conditions (Fig. 1C). Three principal compartment groups were evident: cytosolic and cytoskeletal proteins (fraction 1); membrane-associated proteins, including ER/Golgi and other organelles (fractions 3–4); and nuclear proteins (fractions 2, 5, and 6). Together, these data demonstrate the high reproducibility and resolution of our spatial proteomics pipeline and establish a robust framework to dissect how the FLT3-ITD insertion site governs midostaurin-dependent proteome redistribution and downstream cellular responses, impacting on TKI sensitivity.

Midostaurin-dependent subcellular redistribution of the global proteome in FLT3-ITD cells

Our spatial proteomic workflow enables accurate quantification of proteins across three major subcellular compartments (cytosol,

nucleus, and organelles) in both untreated and midostaurin-treated FLT3-ITD cells (Fig. 1). To systematically identify proteins translocating between compartments, we applied a classification strategy integrating support vector machine (SVM) learning with correlation profiling (Fig. 2A). Using compartment-specific marker profiles to train the SVM (Table S4), we reliably assigned each of the ~8000 quantified proteins to a nuclear, cytosolic, or organellar category in every experimental condition (Fig. 2B; Table S5). The high assignment accuracy (>80% of training proteins correctly classified; Fig. S2C) confirmed the robustness of our approach, while the compartment assignment of known-relocalizing proteins, such as MYC, demonstrated the efficacy of our method (Fig. S2D).

Across all conditions, 2524 proteins changed their subcellular localization in response to midostaurin (Table S6). Of these, ~1065 relocalized specifically in FLT3^{ITD-JMD} cells and 866 in FLT3^{ITD-TKD} cells, while 593 proteins were shared (Fig. 2C). Notably, only one-third of the shared proteins moved to the same destination compartment, whereas two-thirds redistributed in opposite directions (Fig. S2E), underscoring the strong influence of the ITD insertion site on midostaurin-dependent spatial proteome remodeling. We next examined whether specific protein complexes were differentially mobilized between the cytosol and nucleus. Strikingly, FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells displayed profoundly distinct patterns of complex translocation (Fig. 2D–G). In midostaurin-sensitive FLT3^{ITD-JMD} cells, treatment induced extensive nuclear export of complexes critical for cell-cycle progression, DNA replication, and mitosis, including CDK–cyclin modules and the MCM helicase complexes (Fig. 2D, E). Key transcriptional regulators with established roles in AML survival, including NCOR1, RARA, FOS, and TBL1XR1, also redistributed from the nucleus to the cytosol, consistent with disruption of essential survival programs and facilitation of apoptosis [13, 14]. In midostaurin-resistant FLT3^{ITD-TKD} cells, protein-complex translocation was markedly attenuated and qualitatively distinct (Fig. 2F, G). Relocalized proteins were largely restricted to chromatin-remodeling, histone-regulatory, and RNA-stability pathways. Nuclear import of factors involved in the DNA damage response, including ATR-associated components, suggested activation of protective DNA repair programs. Importantly, major complexes extensively mobilized in FLT3^{ITD-JMD} cells, such as those governing replication licensing, mitotic entry, and cell-cycle progression, remained largely static in FLT3^{ITD-TKD} cells.

Genotype-specific autophagy response in midostaurin-treated FLT3-ITD cells

To investigate whether midostaurin-dependent translocating proteins in FLT3-ITD cells were involved in additional specific cellular processes, we performed a Gene Ontology enrichment analysis. Consistently with our data on protein complexes, proteins involved in cell cycle progression, DNA replication and repair were mostly translocated between cytoplasm and nucleus in FLT3^{ITD-JMD} cells and, to a lesser extent, in FLT3^{ITD-TKD} cells. Furthermore, our analysis showed a relocalization of autophagy-related proteins in both FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells following midostaurin treatment (Fig. 3A, B). A more detailed investigation revealed pronounced differences in the subcellular distribution of these proteins between TKI-sensitive and resistant FLT3-ITD cells. In FLT3^{ITD-JMD} cells, midostaurin treatment led to the recruitment of the TFEB–FNIP–FLCN complex and the adaptor protein p62 (SQSTM1) to an organellar compartment, most likely corresponding to lysosomes [15, 16] (Fig. 3C). This observation suggests that midostaurin treatment likely promotes autophagy in FLT3^{ITD-JMD} cells. In contrast, in FLT3^{ITD-TKD} cells, the TFEB–FNIP–FLCN complex and p62 lost their association with these organelles after treatment and instead became diffusely distributed throughout the cytoplasm (Fig. 3C). Moreover, by integrating the subcellular proteomic data with the phosphoproteomic profiles, we selected proteins whose phosphorylation levels changed significantly upon midostaurin treatment and that also displayed a relocalization event, and we merged

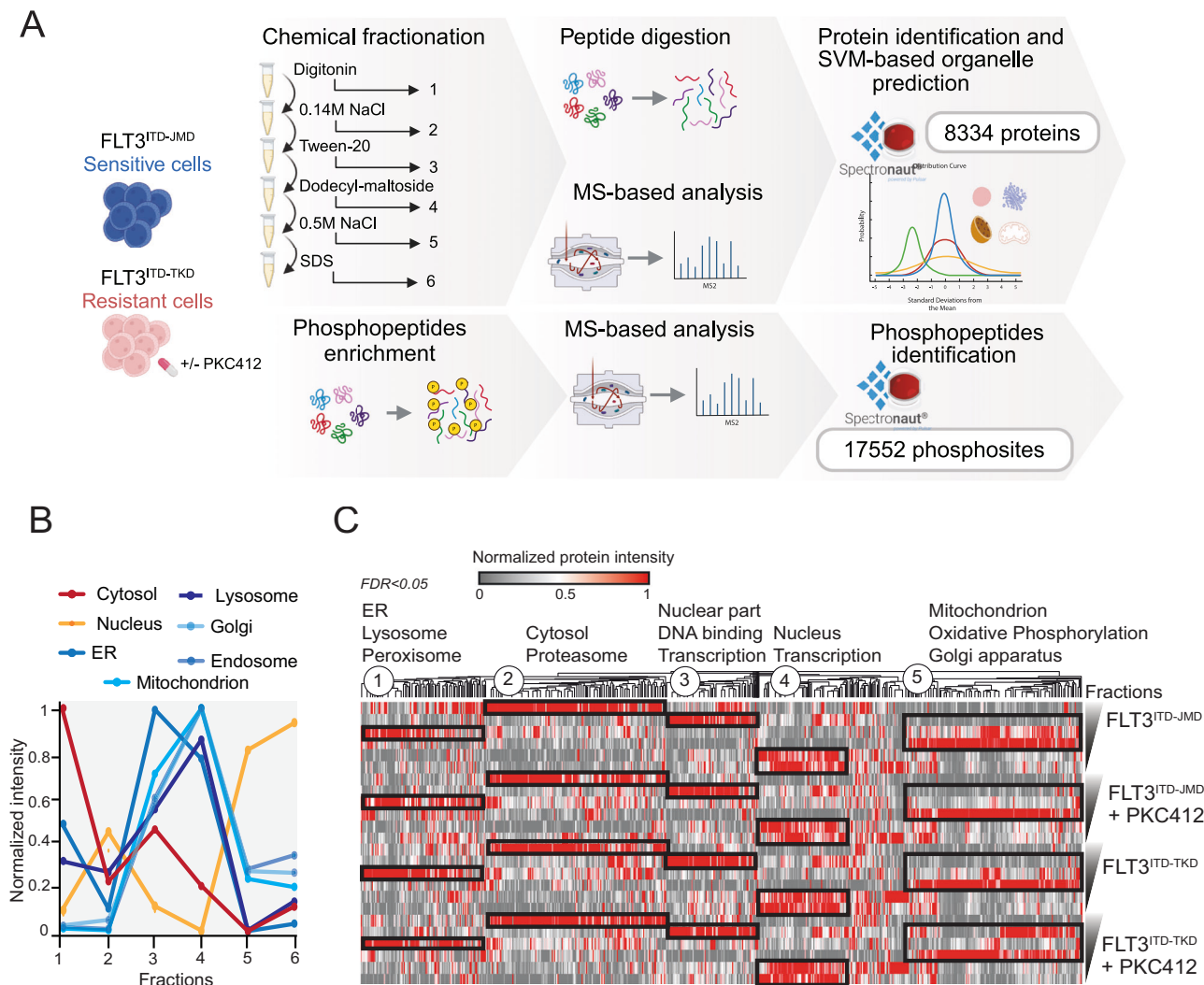


Fig. 1 Organelle proteomics generates midostaurin-specific subcellular maps in FLT3-ITD cells. **A** Schematic overview of the organelle proteomics pipeline. FLT3^{ITD-JMD} (TKI-sensitive) and FLT3^{ITD-TKD} (TKI-resistant) Ba/F3 cells were left untreated or treated with 100 nM midostaurin for 24 h. Sequential extraction with six buffers generated six subcellular fractions, which were analyzed by MS-based proteomics in biological quadruplicates. Peptides were subjected to global proteomic profiling and phosphopeptide enrichment. **B** Intensity profiles of organelle-specific marker proteins across the six fractions. **C** Hierarchical clustering of protein profiles (scaled intensities) across fractions for untreated and midostaurin-treated FLT3-ITD cells. Statistically significant enriched gene ontology terms in each cluster are indicated.

the two datasets to identify overlapping candidates showing coordinated changes in phosphorylation and subcellular distribution (Table S7, Fig. S2F). We found that the autophagy-related proteins displaying differential subcellular localization in FLT3^{ITD-JMD} versus FLT3^{ITD-TKD} cells, also showed opposite phosphorylation patterns after midostaurin treatment (Fig. 3D). Except for TFEB (S466) and PLCN (S62), the regulatory roles of the other phosphosites are currently undefined. Integrating subcellular proteomics with phosphoproteomics, our analysis suggests that these phosphosites may participate in controlling protein intracellular localization. These observations led us to hypothesize that midostaurin treatment differentially affects autophagy in FLT3-ITD sensitive and resistant cells. To this aim, we measured autophagy flux in both sensitive and resistant FLT3-ITD cells treated with midostaurin, either alone or in combination with Bafilomycin A1, an inhibitor of lysosomal V-ATPase. As shown by the accumulation of LC3-II, midostaurin treatment is able to induce autophagy only in FLT3^{ITD-JMD} cells (Fig. 3E). We next investigated whether FLT3^{ITD-TKD} cells are intrinsically defective in autophagy. Thus, FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells were starved for 3 h in the presence or absence of Bafilomycin A1, and autophagy flux was monitored by measuring LC3

lipidation. Both FLT3-ITD cell types showed comparable induction of autophagy upon starvation, indicating that midostaurin specifically affects autophagy in a FLT3 genotype-specific manner (Fig. S3A). As autophagy impairment has been linked to cancer drug resistance [17], we investigated whether autophagy induction could increase the TKI sensitivity of FLT3^{ITD-TKD} cells. To this aim, FLT3-ITD cells were exposed to midostaurin for 24 h, nutrient deprivation for 3 h, or a combination of both treatments. Strikingly, starvation restored the sensitivity of FLT3^{ITD-TKD} cells to midostaurin, suggesting that impaired autophagy may contribute to their resistance phenotype (Fig. 3F). These findings demonstrate that the ITD insertion site influences the capacity of AML cells to activate autophagy in response to midostaurin. Moreover, our data show that short-term nutrient deprivation induces cell death in both FLT3-ITD cell types, highlighting a previously unrecognized potential metabolic vulnerability.

Midostaurin induces ITD site-dependent metabolic reprogramming

Our results show that the ITD insertion site determines the autophagy response to midostaurin and uncover a shared

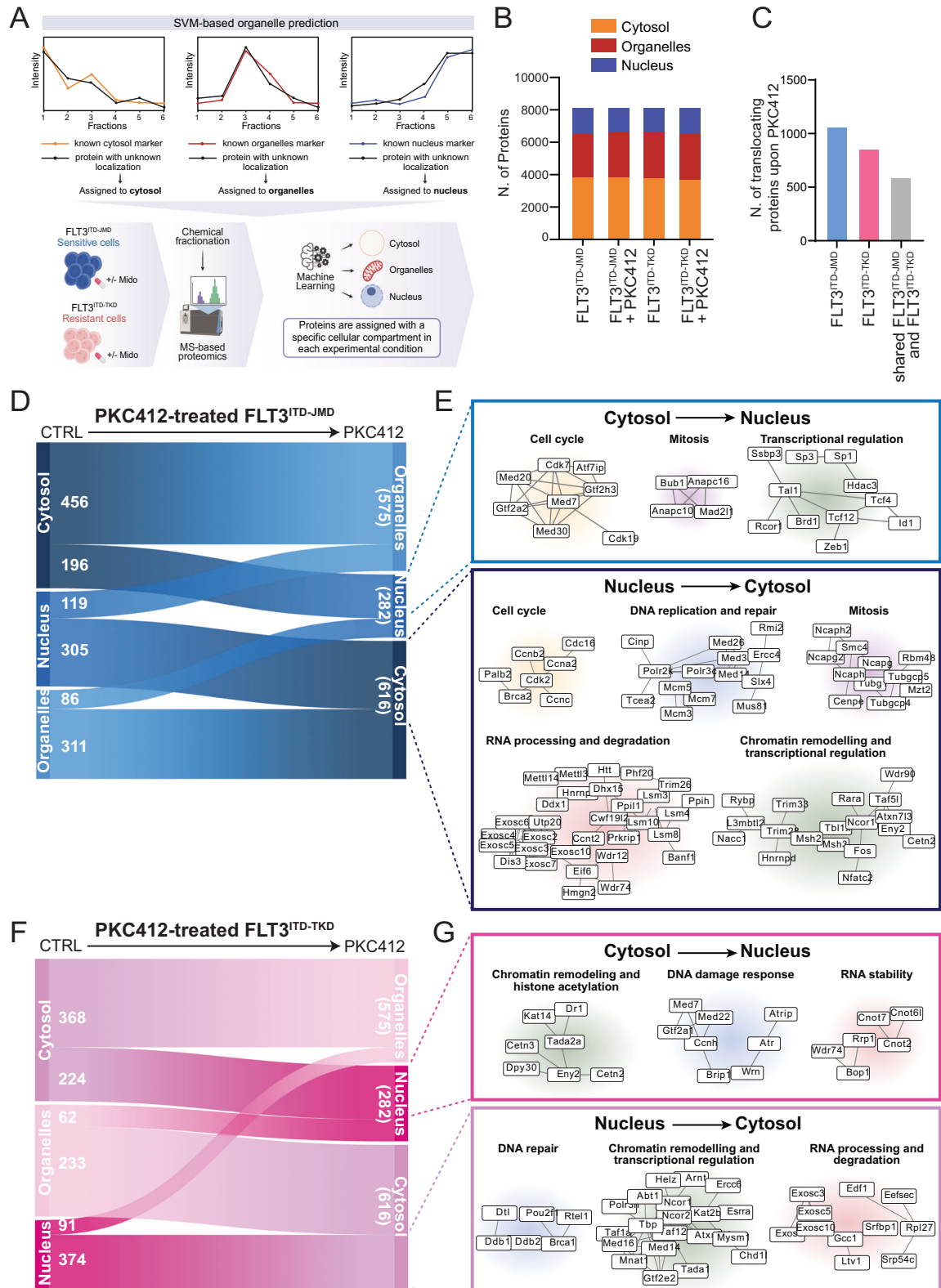


Fig. 2 Midostaurin induces genotype-specific subcellular proteome reorganization in FLT3-ITD cells. **A** Schematic representation of the bioinformatic strategy used to assign a cell compartment to proteins by using the SVM-algorithm. **B** Number of proteins assigned to cytosolic, nuclear, or organellar compartments in untreated and midostaurin treated FLT3-ITD cells. **C** Quantification of proteins changing their subcellular localization upon midostaurin treatment. **D** Sankey plot of protein translocation in midostaurin-treated FLT3^{ITD-JMD}. **E** Functional protein complexes redistributed between cytosol and nucleus in midostaurin-treated FLT3^{ITD-JMD}. **F** Sankey plot of protein translocation in midostaurin-treated FLT3^{ITD-TKD} cells. **G** Functional protein complexes redistributed between cytosol and nucleus in midostaurin-treated FLT3^{ITD-TKD} cells.

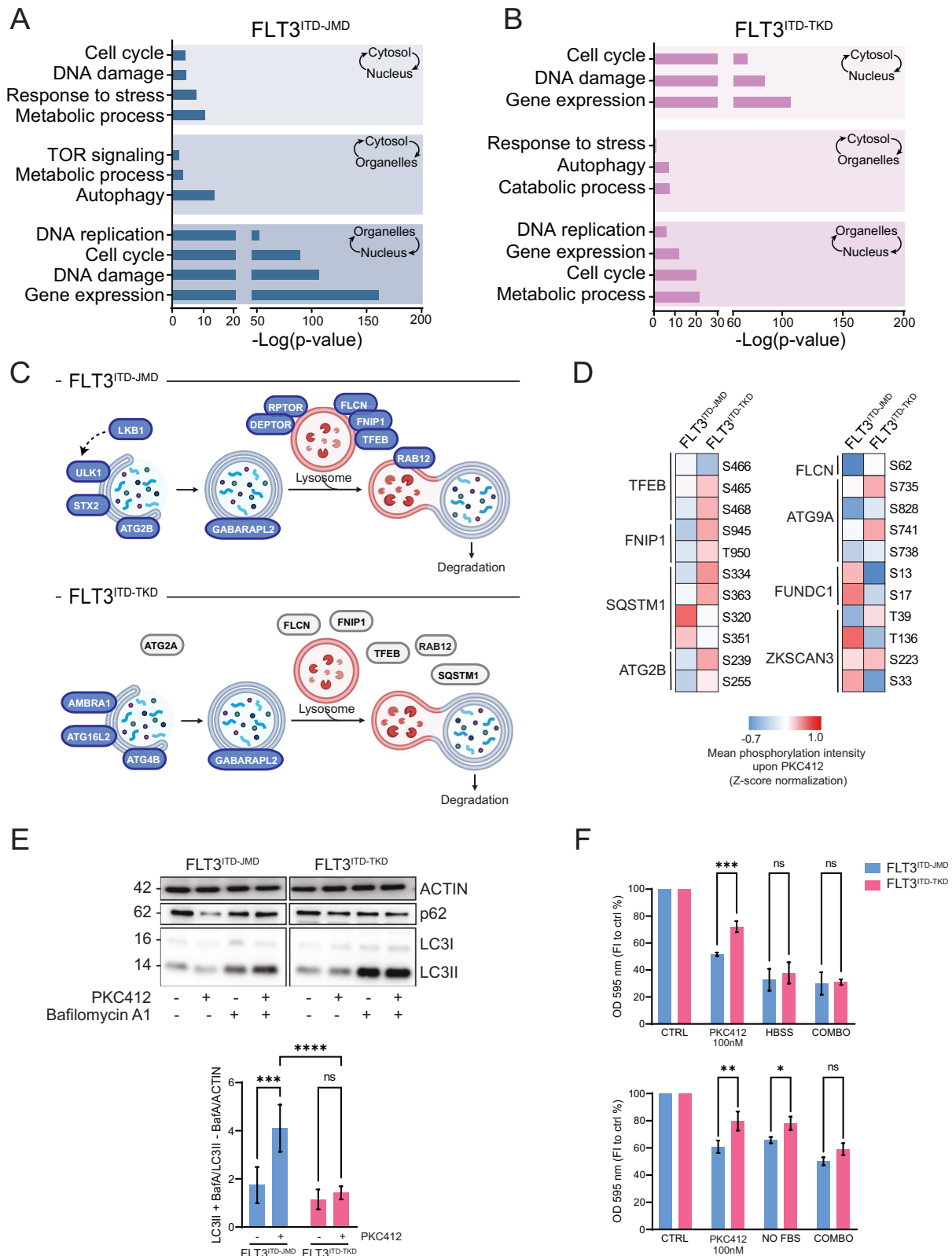


Fig. 3 Midostaurin-induced mitochondrial remodeling and spatial changes. **A, B** Bar plots reporting the $-\log_2(p\text{-value})$ of the GO-Term enrichment analysis of translocating proteins upon midostaurin treatment in sensitive and resistant FLT3-ITD cells. **C** Schematic representation of the spatial remodeling of key autophagic proteins upon midostaurin treatment in sensitive and resistant FLT3-ITD cells. **D** Heatmap reporting the intensity of the phosphorylation level of translocating autophagic proteins after midostaurin treatment in FLT3^{ITD-JMD} and FLT3^{ITD-TKD}. **E** Western blotting of LC3-II and p62 protein levels in FLT3^{ITD-JMD} and FLT3^{ITD-TKD}. Cells were treated with midostaurin with or without bafilomycin A1 for 24 h. **F** Bar plot reporting the percentage of viable cells measured by MTT assay. FLT3^{ITD-JMD} and FLT3^{ITD-TKD} were treated with 100 nm of midostaurin treatment for 24 h either alone or combined with 3 h of starvation.

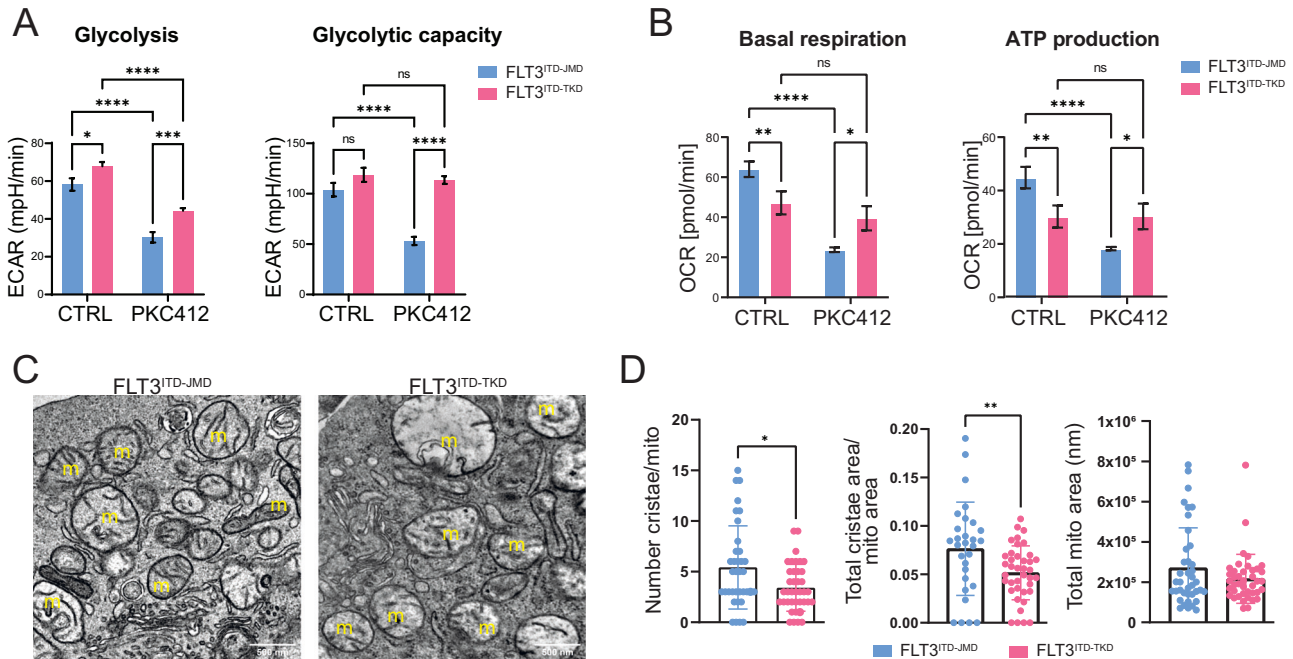


Fig. 4 Midostaurin induces ITD site-dependent metabolic reprogramming. **A** Bar plot reporting the ECAR based measurements of glycolysis and glycolytic capacity in FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells. Cells were treated with 100 nM midostaurin for 24 h and subjected to a glycolysis stress test. **B** OCR-derived assessments of basal respiration and ATP production in FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells. Cells were treated with 100 nM midostaurin for 24 h and subjected to a mitochondrial stress test. **C** Representative electron microscopy images of steady-state FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells (m=mitochondria). **D** Bar plots reporting the number of cristae, the total cristae area and total mitochondria area in steady-state FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells.

vulnerability of both FLT3-ITD cell types to short-term nutrient deprivation. Building on these observations, we next investigated how ITD location influences the metabolic response to midostaurin. To this end, FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells were treated with midostaurin for 24 h or left untreated and their glycolytic and oxidative phosphorylation activities were assessed using Seahorse extracellular flux analysis. In control condition, FLT3^{ITD-JMD} cells displayed a slightly higher glycolysis and glycolytic capacity than FLT3^{ITD-TKD} cells, as indicated by elevated extracellular acidification rate (ECAR) (Fig. 4A). This metabolic profile was further supported by significantly elevated basal respiration and ATP production in FLT3^{ITD-JMD} cells compared to FLT3^{ITD-TKD} cells (Fig. 4B). Consistently, transmission electron microscopy (TEM) images revealed that FLT3^{ITD-JMD} cells appear to have a higher number of cristae and an increased cristae-to-mitochondrial ratio compared to FLT3^{ITD-TKD} cells, a morphology in line with their higher basal respiration and ATP production (Fig. 4C, D). Following midostaurin (PKC412) treatment, FLT3^{ITD-JMD} cells displayed a strong reduction in glycolysis, glycolytic capacity, basal respiration and ATP production, while FLT3^{ITD-TKD} cells did not exhibit significant metabolic changes (Fig. 4A, B). Consistently, phosphorylation levels of PKM2 Y105 decreased upon midostaurin treatment only in FLT3^{ITD-JMD} cells, but not in FLT3^{ITD-TKD} cells (Fig. S3B). Together, these results demonstrate that the ITD insertion site influences key metabolic processes already under steady-state conditions and highlights the metabolically TKI-resistant phenotype of FLT3^{ITD-TKD} cells.

Next, we leveraged our subcellular proteomics dataset to examine how mitochondrial proteins distribute across fractions. We focused on the 168 proteins annotated as “mitochondrial” in the MitoCarta database [18–20] and analyzed their abundance profiles across the six fractions (Table S8). Unsupervised clustering identified three major groups enriched for mitochondrial matrix proteins, mitochondrial small ribosomal subunits, and oxidative phosphorylation (OXPHOS) complexes (Fig. 4E). Mitochondrial

matrix proteins and small ribosomal subunit proteins displayed highly similar distribution profiles in untreated and midostaurin-treated FLT3-ITD cells, indicating that these mitochondrial components are not substantially affected neither by TKI exposure nor by the ITD insertion site (Fig. 4E). In contrast, OXPHOS enzymes showed a markedly different behavior (Fig. S3C, D) only in midostaurin-treated FLT3^{ITD-TKD} cells, exhibiting a prominent enrichment in fraction 2. We initially hypothesized that this observation could be explained by an increased mitochondria–nucleus contact sites. However, immunofluorescence analysis revealed no co-localization between the mitochondrial marker TOMM20 and nuclear staining, arguing against this interpretation (Fig. S4A). Instead, the finding is more consistent with the higher mitotic rate of FLT3^{ITD-TKD} cells upon TKI treatment compared to FLT3^{ITD-JMD} [6]. During mitosis, nuclear envelope breakdown allows proteins from distinct subcellular compartments, including mitochondrial proteins, to co-fractionate with the nuclear fraction (Fraction 2). Notably, similar co-purification patterns have been previously reported using the same fractionation approach [10].

Lipid-dependent mitochondrial activity in TKI treated FLT3^{ITD-TKD} cells

Our results reveal that FLT3^{ITD-TKD} cells are metabolically unresponsive to TKI treatment (Fig. 4A, B) and can be re-sensitized to TKI by short-term nutrient deprivation (Fig. 3F). These findings prompted us to investigate which metabolic substrates sustain mitochondrial respiration in FLT3^{ITD-TKD} cells under midostaurin treatment. Subcellular proteomics showed that both FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells exhibit a strong enrichment in enzymes involved in lipid metabolism translocating from cytosol to organelles (Fig. 5A), suggesting alternative substrate utilization.

To investigate how FLT3-ITD cells fuel mitochondrial respiration, we examined changes in oxygen consumption rate (Δ OCR) following inhibition of glutamine oxidation (BPTES), fatty-acid β -

oxidation (Etomoxir), and pyruvate import (UK5099) in FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells, with or without midostaurin treatment (Fig. 5B). As previously shown (Fig. 4A, B), midostaurin markedly suppresses ATP production in FLT3^{ITD-JMD} cells, whereas FLT3^{ITD-TKD} cells remain metabolically insensitive. Inhibition of mitochondrial fuel pathways, in absence of midostaurin, revealed distinct metabolic features of FLT3^{ITD-TKD} cells. Blockade of glutamine oxidation (BPTES) and pyruvate import (UK5099) resulted in a stronger Δ OCR reduction in FLT3^{ITD-JMD} compared with FLT3^{ITD-TKD} cells, suggesting a higher dependence on glucose and glutamine metabolism in the JMD context. Fatty-acid oxidation inhibition causes a clear decrease in Δ OCR in both genotypes (Fig. 5B, upper panel). Conversely, in PKC412 treated cells, etomoxir produces a divergent response: Δ OCR significantly decreases in FLT3^{ITD-TKD} cells, while FLT3^{ITD-JMD} cells display a compensatory increase in respiration (Fig. 5B, lower panel), demonstrating that FLT3^{ITD-TKD} cells remain critically dependent on fatty-acid oxidation to sustain mitochondrial activity when FLT3 signaling is inhibited.

Lipid availability modulates FLT3-ITD localization and TKI response

To further explore whether altered fatty-acid metabolism contributes to the differential TKI sensitivity of FLT3-ITD variants, we assessed cell viability under nutrient-restricting conditions, by the removal of glucose, amino acids, or lipids, alone or in combination with midostaurin. Specifically, FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells were incubated with or without midostaurin in a medium deprived of glucose, amino acids or lipids for 24 h and the percentage of viable cells was reported. As shown in Fig. 5C, FLT3^{ITD-JMD} cells displayed a substantial decrease in viability upon midostaurin treatment, and this effect was not impacted when glucose or amino acids were removed. In contrast, FLT3^{ITD-TKD} cells preserved their intrinsic resistance to TKI treatment and remained largely insensitive to midostaurin under glucose or amino-acid deprivation. However, lipid deprivation produced a different pattern: although removal of lipids alone increased cell viability in both genotypes, its combination with midostaurin specifically abolished midostaurin resistance in FLT3^{ITD-TKD} cells, resulting in a pronounced viability loss (Fig. 5C). These findings are consistent with our metabolic analyses showing that, upon FLT3 inhibition, FLT3^{ITD-TKD} cells heavily rely on fatty-acid oxidation to sustain residual mitochondrial respiration (Fig. 5B). Accordingly, restricting lipid availability removes essential metabolic support and selectively restores TKI sensitivity in FLT3^{ITD-TKD} cells. Together, these findings indicate that fatty-acid metabolism represents a crucial metabolic axis in FLT3-ITD cells, and that, although FLT3^{ITD-TKD} cells are generally metabolically resistant to TKI treatment, their survival becomes markedly compromised when lipid availability is restricted. This prompted us to investigate more closely how lipid deprivation affects FLT3-ITD biology.

FLT3-ITD receptors are known to undergo S-palmitoylation at Cys563 by the ER-resident palmitoyl acyltransferase ZDHHC6 [21]. Unlike wild-type FLT3, which traffics efficiently to the plasma membrane, FLT3-ITD accumulates predominantly in the ER and only weakly reaches the cell surface [22]. We therefore asked whether lipid deprivation could influence FLT3-ITD cell viability by altering palmitoylation-dependent trafficking and thereby modifying FLT3 subcellular localization.

To test this, we examined FLT3 localization by immunofluorescence in FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells cultured in control (CTRL) or lipid-depleted serum (LD-FBS). Cells were stained either without permeabilization, to assess cell-surface FLT3, or with permeabilization, to visualize total intracellular FLT3 (Fig. 5D). Under control conditions, both FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells displayed minimal FLT3 signal at the plasma membrane, consistent with the well-described intracellular retention of FLT3-ITD. Strikingly, lipid deprivation caused a robust increase in cell-surface FLT3 in both genotypes, with a prominent ring-like staining pattern more

pronounced in FLT3^{ITD-TKD} cells but clearly visible in FLT3^{ITD-JMD} cells as well. This is likely due to an impairment of FLT3 palmitoylation events. Indeed, inhibition of palmitoylation with 2-bromopalmitate (2-BP) recapitulated the same phenotype (Fig. S4B). Because cell-surface FLT3 is known to have stronger oncogenic potential than its ER-retained counterpart [22], the increased membrane localization observed under lipid-deprived conditions is consistent with the enhanced viability seen in the absence of midostaurin. Importantly, by performing flow cytometry analysis of total and surface FLT3, we confirmed that lipid starvation also increases FLT3 levels at the plasma membrane in primary AML blasts carrying FLT3-ITD mutations, extending our observations beyond cell lines (Fig. 5E). In contrast, when lipid deprivation was combined with midostaurin treatment, FLT3-ITD cells exhibited a marked reduction in viability (Fig. 5C). This finding suggests that lipid-mediated redistribution of FLT3^{ITD-TKD} to the plasma membrane increases receptor accessibility to midostaurin, thereby restoring TKI sensitivity. Collectively, these data indicate that lipid-dependent compartmentalization of FLT3-ITD governs its membrane exposure and shapes the differential TKI responsiveness of FLT3-ITD cells, restoring sensitivity in FLT3^{ITD-TKD} cells while also influencing midostaurin-sensitive populations.

Importantly, although beyond the scope of the present study, we decided to validate this lipid-dependent modulation of TKI response *ex vivo*. To this end, we used primary AML blasts carrying different FLT3-ITD mutations: FLT3^{ITD-TKD} blasts (carrying the ITD in the TK domain), FLT3^{ITD-JMD+ITD-TKD} blasts (carrying the ITD in both the JM and TK domain), and FLT3^{ITD-JMD} blasts (carrying the ITD in the JM domain). Our experiments showed that lipid deprivation in combination with midostaurin led to a marked increase in cell death in all the FLT3-ITD samples, confirming the translational relevance of lipid dependency in primary leukemic cells (Fig. 6A). However, it is important to highlight that lipid deprivation alone exerts opposite effects between Ba/F3 cells and primary AML blasts. This discrepancy likely reflects intrinsic biological differences between the models. Ba/F3 cells strongly overexpress ectopic FLT3-ITD constructs, a feature that may substantially alter downstream signaling and metabolic dependencies, potentially masking metabolic stress responses. In contrast, primary AML blasts represent a more physiologically relevant context, in which endogenous FLT3-ITD expression levels and lineage-specific metabolic programs may confer heightened sensitivity to lipid availability. Collectively, our findings indicate that fatty-acid metabolism represents a critical metabolic axis in FLT3-ITD-driven leukemia and that lipid availability broadly influences TKI responsiveness across FLT3-ITD subtypes, uncovering a shared, lipid-dependent vulnerability that may be therapeutically exploitable (Fig. 6B).

DISCUSSION

FLT3-ITD mutations are among the most important genetic determinants of prognosis and therapeutic response in AML [23]. Midostaurin in combination with chemotherapy has improved outcomes for FLT3-mutated patients, however a significant subset, especially those harboring ITDs within the TKD region, continues to exhibit primary resistance and poor survival [5].

Our study revealed that the FLT3-ITD insertion site affects how leukemic cells respond to kinase inhibition, by coordinating changes in protein localization, phosphorylation, autophagy, and metabolic plasticity. By using high-resolution subcellular proteomics, we show that midostaurin induces a profound reorganization of the proteome, affecting the localization of more than 2500 proteins. The functional consequences of such huge proteome remodeling are strongly dictated by the ITD insertion site. In FLT3^{ITD-JMD} cells, proteins critical for DNA replication, cell-cycle progression and mitosis (such as CDK-cyclin complexes, the MCM

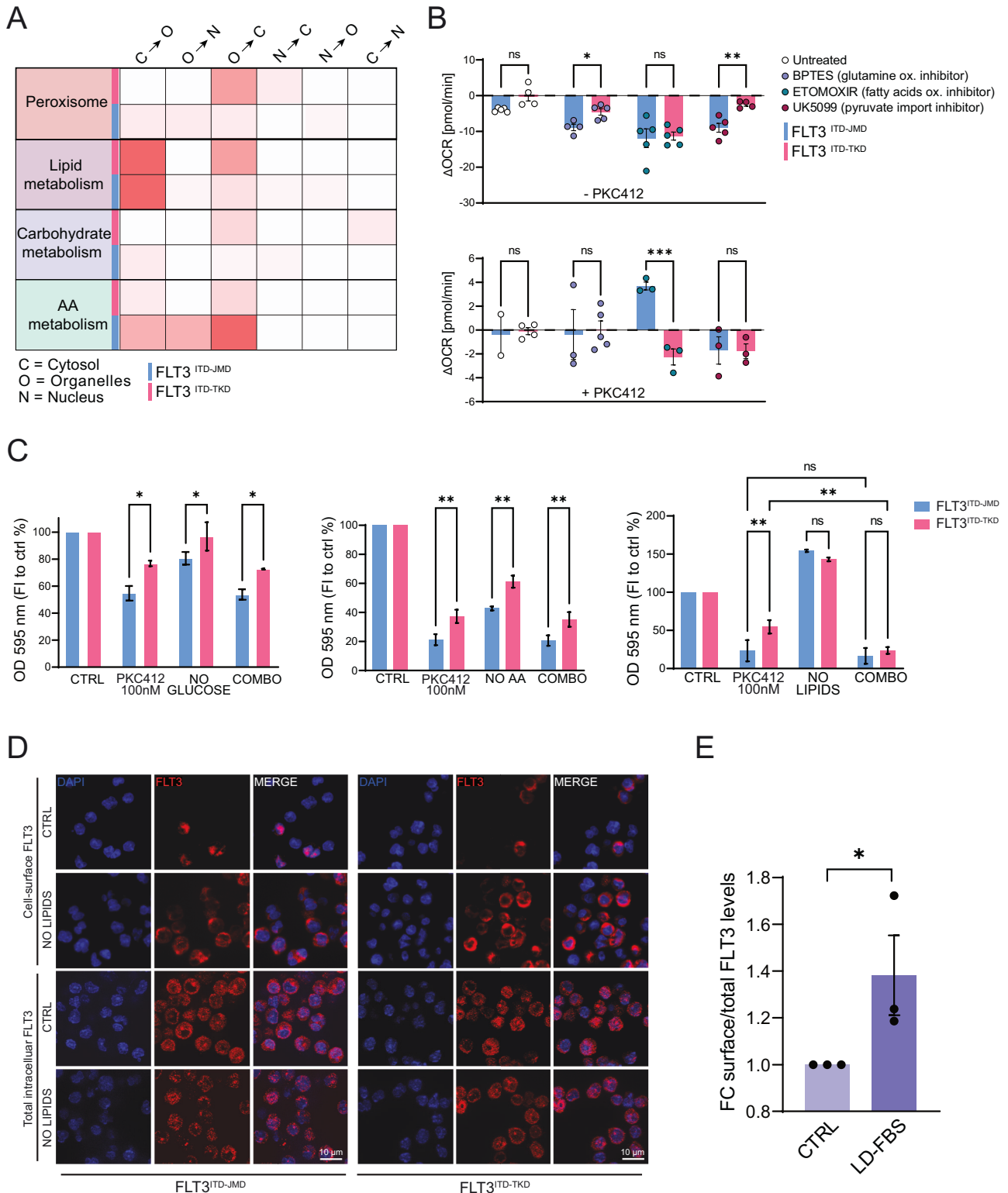


Fig. 5 Lipid availability modulates FLT3-ITD localization and TKI response. **A** Heatmap reporting the number of translocating proteins involved in carbohydrate, amino acids, peroxisome and lipid metabolism in FLT3^{ITD-JMD} and FLT3^{ITD-TKD}. **B** Bar plot reporting the Δ OCR of basal respiration after the inhibition of glucose usage (UK5099), glutamine usage (BPTES) and lipid usage (ETOMOXIR) for OXPHOS in FLT3^{ITD-JMD} and FLT3^{ITD-TKD} in absence and presence of 100 nM midostaurin treatment for 24 h. **C** Bar plot reporting the percentage of viable cells measured by MTT assay. FLT3^{ITD-JMD} and FLT3^{ITD-TKD} were treated with 100 nm of midostaurin treatment for 24 h either alone or combined with glucose, amino acid and lipid deprivation. **D** Representative immunofluorescence images of FLT3 localization before and after lipid deprivation in both permeabilized and non-permeabilized FLT3^{ITD-JMD} and FLT3^{ITD-TKD}. **E** Bar plot reporting the percentage of FLT3 surface level in four primary FLT3-ITD AML cells in basal condition and upon 24 h of lipid-deprivation.

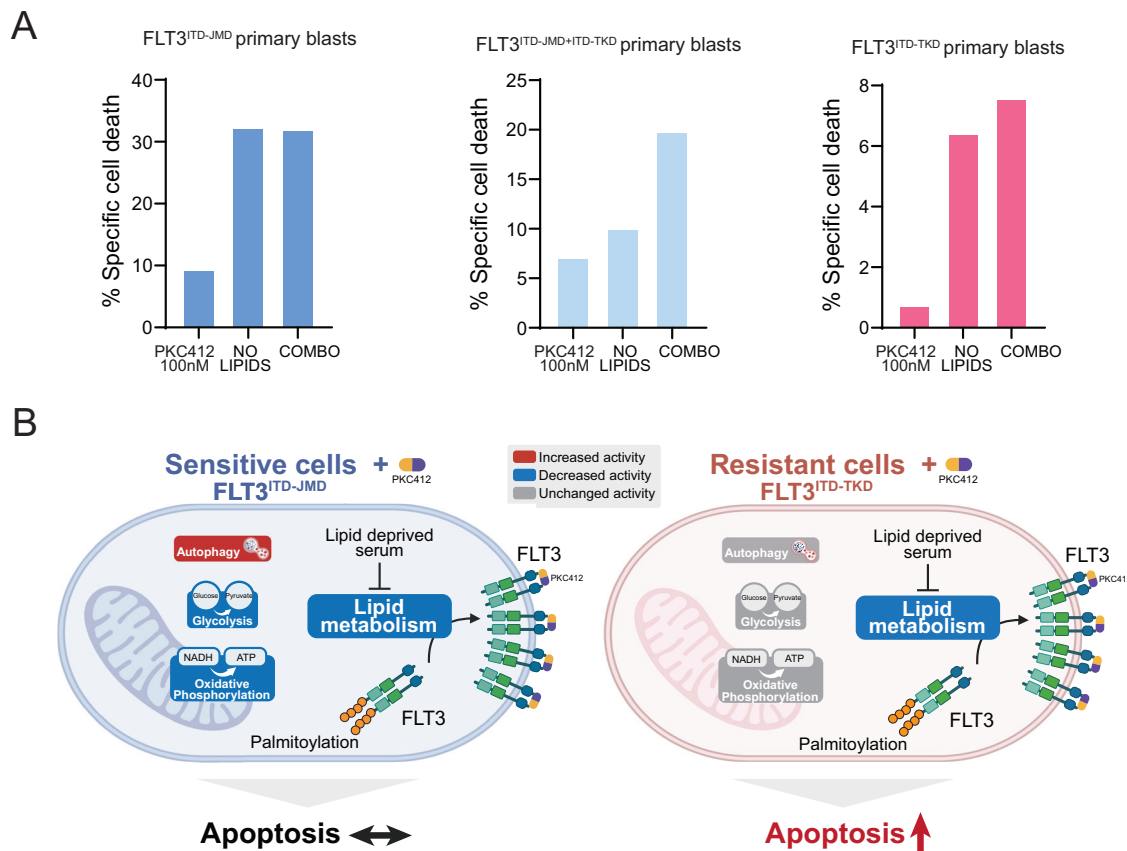


Fig. 6 Lipid availability modulates TKI response in patient-derived FLT3-ITD blasts. **A** Barplots showing the percentage of treatment-induced apoptosis ($100 \times (\text{dead cells after treatment} - \text{death cells in control}) / \text{viable cells in control}$) in patient-derived blasts carrying FLT3-ITD in (i) the JM domain, (ii) both the JM and TK domain and (iii) the TK domain upon the indicated treatments. Percentage of apoptotic cells was assessed by Annexin-V labeling. **B** Graphic model representing the metabolic adaptation and vulnerability of FLT3-ITD cells in response to PKC412 treatment.

helicase, and transcriptional regulators) undergo extensive nuclear export, contributing to cell cycle arrest and apoptosis [24]. In contrast, FLT3^{ITD-TKD} cells exhibit a different pattern of protein mobilization, including the recruitment of chromatin-modifying and DNA repair proteins to the nucleus, suggesting activation of protective stress-repair response. Notably, these observations are largely consistent with previous findings reported for these FLT3-ITD cellular models, confirming the distinct biological behaviors associated with JMD and TKD mutations [8]. However, our study adds a novel layer of information, demonstrating that these functional differences are accompanied by profound changes in the subcellular localization and nucleo-cytoplasmic translocation of key regulatory proteins. This spatial reorganization of proteins involved in cell-cycle control, chromatin regulation, and DNA damage response provides mechanistic insight into how FLT3^{ITD-JMD} and FLT3^{ITD-TKD} cells differentially adapt to TKI treatment.

We acknowledge that fractionation-based spatial proteomics does not achieve perfect separation of all organelles, and that some apparent protein relocalizations may reflect partial overlap between fractions rather than absolute organelle translocation. To mitigate this, we applied a replicate-consistency filter, considering only proteins showing reproducible redistribution across at least three out of four biological replicates. Consequently, our conclusions focus on relative redistribution patterns and coordinated pathway-level effects, rather than asserting absolute compartment switching for individual proteins. Importantly, proteins with well-established organelle localization, such as mitochondrial matrix and ribosomal proteins, remained largely stable, confirming the robustness and specificity of our approach. This strategy allows us

to reliably identify biologically meaningful subcellular remodeling events induced by midostaurin in a FLT3-ITD context, while transparently acknowledging the methodological limitations.

One of the most striking findings of our study is that autophagy response to midostaurin is ITD-dependent. Indeed, midostaurin triggers robust autophagy activation in FLT3^{ITD-JMD} cells, as observed by organellar recruitment of TFEB, FNIP/FLCN and p62, together with increased LC3 lipidation. This pattern is consistent with the well-established mechanism whereby the FNIP/FLCN complex is dynamically recruited to lysosomal membranes, regulating TFEB activation in response to metabolic or lysosomal stress [15]. In contrast, FLT3^{ITD-TKD} cells fail to activate autophagy upon midostaurin treatment. Notably, this observation differs from previous reports which identified autophagy as a resistance mechanism to FLT3 inhibition [25]. This apparent discrepancy may reflect differences in the type of inhibitor used, as midostaurin is a multi-kinase inhibitor, as well as differences in cellular context and experimental approaches used to assess autophagy. Notably, our findings are consistent with recent evidence linking defective autophagy signaling to drug resistance in multiple cancer types and further support the notion that resistance mechanisms to FLT3-targeting agents are highly context-dependent [26].

Moreover, our study indicates that ITD location remodels cellular metabolism already under steady-state conditions. Transmission electron microscopy (TEM) combined with metabolic assays revealed that FLT3^{ITD-JMD} cells harbor a greater number of mitochondria that are larger and richer in cristae compared with FLT3^{ITD-TKD} cells, consistent with their higher basal respiration and ATP production [27, 28]. The molecular mechanisms driving this

ITD-dependent mitochondrial and metabolic remodeling remain unknown and require further investigation. In line with their differential sensitivity to TKI treatment, FLT3^{ITD-JMD} cells undergo a marked reduction in both glycolysis and oxidative phosphorylation upon midostaurin exposure, coupled with mitochondrial condensation and a consequent drop in ATP levels. By contrast, FLT3^{ITD-TKD} cells maintain stable glycolytic and respiratory activity, displaying a metabolically resilient phenotype.

Integrating our subcellular proteomic dataset with metabolic analyses, we identified lipid metabolism as a potential critical determinant of TKI resistance. Our data show that lipid deprivation, but not amino acid or glucose deprivation, sensitizes FLT3^{ITD-TKD} cells to midostaurin treatment. We hypothesize that this lipid-dependent modulation of TKI sensitivity reflects a direct effect on FLT3. FLT3-ITD palmitoylation by the ER-resident acyltransferase ZDHHC6 promotes receptor retention within the ER [21]. Our working model proposes that lipid deprivation reduces palmitoylation efficiency, thereby increasing FLT3 surface exposure. As cell-surface FLT3 possesses higher oncogenic activity and enhances proliferation, its redistribution under lipid-depleted conditions is consistent with the increased viability observed in the absence of midostaurin in cell line models. However, increased membrane localization also renders the receptor more accessible to TKIs, resulting in enhanced cell death in resistant FLT3^{ITD-TKD} cells. Importantly, we validated the lipid vulnerability of FLT3-ITD cells in ex vivo experiments using primary AML blasts. Importantly, ex vivo experiments using primary AML blasts revealed that lipid deprivation alone is sufficient to induce cell death, highlighting an intrinsic lipid dependency of FLT3-ITD-positive blasts. However, given the small number of patient samples included, our findings will require additional confirmation, which lies beyond the scope of the present study. Lipid deprivation has previously been implicated in FLT3-ITD-driven leukemia [29, 30]. This resulted in clinical trials assessing the potential benefit of introducing low-fat diet regimen or statins to chemotherapy treatment [28]. Accordingly, our findings demonstrated that lipid-dependent compartmentalization of FLT3-ITD governs therapeutic vulnerability. However, our data also suggest that lipid deprivation may amplify additional metabolic and signaling vulnerabilities beyond the alteration of receptor localization. In particular, restriction of lipid availability could impair mitochondrial membrane composition and electron transport chain efficiency or affect post-translational lipid modifications of mitochondrial proteins, thereby perturbing pro-survival signaling networks [31–33]. Moreover, lipid-deprived conditions may induce changes in membrane lipid composition and, thus, influence receptor clustering, endocytosis, and downstream signaling pathways, increasing sensitivity to TKI treatment [34]. Dissecting these interconnected lipid-dependent mechanisms will be essential to fully understand how lipid restriction synergizes with TKIs to overcome resistance. Collectively, our data support the notion that FLT3-ITD insertion site is a fundamental determinant of disease heterogeneity in AML, as it drives distinct signaling programs, proteome remodeling, metabolic adaptations, and differential responses to kinase inhibition, consistent with clinical observations [5]. The coupling of spatial proteomics with phospho-proteomics offers a powerful framework for dissecting subcellular drug effects and identifying compartment-specific resistance mechanisms in oncogenic signaling.

DATA AVAILABILITY

The mass spectrometry-based proteomics data have been deposited at the ProteomeXchange Consortium, via the PRIDE partner repository (n. PXD073478).

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AUTHOR CONTRIBUTIONS

V.B. and A.F.P. were responsible for the conceptualization of the study together with G.M., L.P. and F.S. The methodology was designed by V.B., A.F.P. and G.M., with contributions from S.G., M.B., L.P., F.N., V.M., M.C. and F.S. Formal analysis was carried out by V.B., A.F.P. and G.M., with the involvement of S.G., M.B., F.N., V.M. and M.C. The investigation was conducted by V.B. and A.F.P., with contributions from T.F., M.B. and D.M. V.B., A.F.P., G.M. and F.S. prepared the original draft of the manuscript. All authors contributed to reviewing and editing the manuscript. Supervision was provided by G.M. and F.S., while F.S. was responsible for funding acquisition. All authors have read and agreed to the published version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods were performed in accordance with the relevant guidelines and regulations. Peripheral blood (PB) samples from AML patients were obtained upon patient's informed consent and in accordance with the declaration of Helsinki (ethics committee approval number: 115/08). Informed consent was obtained from all participants prior to their inclusion in the study.

ADDITIONAL INFORMATION

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