

Review

Targeting Oncogenic Signaling in Mutant FLT3 Acute Myeloid Leukemia: the Path to Least Resistance

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Abstract: Identification of recurrent driver mutations in genes encoding tyrosine kinases has resulted in the development of molecularly targeted strategies designed to improve the outcomes for patients diagnosed with acute myeloid leukemia (AML). The receptor tyrosine kinase FLT3, is the most commonly mutated gene in AML, with internal tandem duplications within the juxtamembrane domain (FLT3-ITD) or missense mutations in the tyrosine kinase domain (FLT3-TKD), present in 30%-35% of AML patients at diagnosis. An established driver mutation and marker of poor prognosis, the FLT3 tyrosine kinase has emerged as an attractive therapeutic target, and thus has encouraged the development of FLT3 tyrosine kinase inhibitors (TKIs). However, the therapeutic benefit of FLT3 inhibition, particularly as monotherapy, frequently results in the development of treatment resistance and disease relapse. Commonly, FLT3 inhibitor resistance is induced by the emergence of secondary lesions in the *FLT3* gene, particularly in the second tyrosine kinase domain at residue Asp835 (D835) to form a 'dual mutation' (ITD-D835). Individual FLT3-ITD and FLT3-TKD mutations influence independent signaling cascades however, currently little is known which divergent signaling pathways are controlled by each of these FLT3 specific mutations, particularly in the context of patients harboring dual ITD-D835 mutations. This review provides a comprehensive analysis of the known discrete and cooperative signaling pathways regulated by each of the FLT3 specific mutations, as well as the therapeutic approaches that hold the most promise for development of more durable and personalized therapeutic approaches targeting mutant FLT3, to improve the treatment of AML.

Keywords: Acute Myeloid Leukemia; FLT3; Tyrosine kinase inhibitors; resistance;

1. Introduction

Acute myeloid leukemia (AML) is characterized by the malignant transformation of a hematopoietic stem/progenitor cell (HSC). This occurs following the acquisition of somatic driver mutations that cooperate with accrued passenger mutations, or lesions that coincidentally occur following the acquisition of the driver mutations [1]. The malignant precursor cells accumulate in the bone marrow and blood at the expense of healthy blood cells, leading to the acute symptoms including anemia, bleeding and bruising, infections and bone pain.

AML is the most common form of acute leukemia in adults and the second most common leukemia in children [2]. It is generally considered a disease of the elderly, with a median age at

diagnosis of 67 years [3]. Elderly patients are characterized by a more genotypically complex disease and are often unable to receive intensive chemotherapy resulting in a particularly bleak prognosis, and are only predicted to survive for 5 to 10 months post diagnosis [4]. Although AML only accounts for 20% of children diagnosed with leukemia, it is responsible for over half of all pediatric leukemia deaths [5]. The disease can emerge as a primary (*de novo* AML), a pre-leukemia (such as myelodysplastic syndrome - MDS), or can be induced following chemotherapy, radiation therapy, immunosuppressive therapy, or a combination of these used to treat pre-existing conditions [6].

Advances in genomic sequencing techniques and technologies have identified recurrent mutations, which have begun to help elucidate the complex genomic landscapes underpinning the disease, both at diagnosis and following relapse [7, 8]. Importantly, these studies have begun to associate individual mutations, and combinations of mutations, with overall survival (OS) [9]. Whole gene sequencing analysis has revealed that mutations are common in signaling genes that encode for the tyrosine kinases, *FLT3*, *JAK2*, *ckIT*; phosphatases, *PTPN11*, *PTPRT*, *PTPN14*; and Ras GTPases, *KRAS* and *NRAS*, and represent 59% of all gene mutations [8, 9]. These are often independently associated with poor outcomes [10]. The commonality of these mutations, particularly to tyrosine kinases, make them attractive molecular targets; however as it stands, targeting individual mutations using precision therapies has failed to deliver the portended increased patient survival.

Oncogenic mutations to the FMS-like tyrosine kinase 3 (FLT3) receptor occur in 30 – 35% of all AML cases at diagnosis. Mutations in FLT3 can manifest as a duplication of a fragment within the juxtamembrane domain coding region (exons 14 and 15), causing an internal tandem duplication (FLT3-ITD) [11]; or as missense mutations in the activation loop (exon 20) of the tyrosine kinase domain (FLT3-TKD), most commonly at aspartic acid 835 (D835; Figure 1) [12]. Both are associated with poor disease-free survival (DFS) and a distinct gene-expression signature [13, 14].

Despite promising initial results, FLT3 inhibitors have shown non-durable anti-leukemic activity as single-agents, with progression seen in virtually all patients [15]. In general, the durability of response tends to be only weeks to months, with long-term efficacy compromised by primary or secondary acquired resistance [16, 17]. Frequently, drug resistance emerges through the development of FLT3-ITD point mutations [18] or TKD mutations [19] following treatment with tyrosine kinase inhibitors (TKIs). Recent clinical trials have demonstrated increased DFS with FLT3 inhibitors in combination with chemotherapy [20], suggesting that combination targeted strategies are required for sustained FLT3 inhibition. Indeed, in 2017 the FDA approved the FLT3 inhibitor, Midostaurin in combination with standard of care chemotherapy for treatment of newly diagnosed FLT3-mutant AML patients [21], superseding an era of very little advancement in AML therapy [22].

Despite the recurrence of FLT3 activating mutations in AML, the differences in oncogenic pathways in patients harboring either ITD or TKD mutations still requires clarification. The significant advancements recently made in unbiased quantitative phosphoproteomic profiling [23–26] has the potential to provide us with a full annotation of the signal pathways regulated in response to individual mutations, or following acquired resistance induced by ‘dual lesions’ (e.g. FLT3-ITD-TKD) [27]. Herein, we review the known oncogenic signaling pathways modulated in FLT3 mutant AML cells, and the roles each of the different FLT3 mutations play in the emergence of targeted-therapy resistance following the use of first and second generation TKIs. A fundamental understanding of the unique and redundant signaling pathways associated with each individual, or dual mutations, holds promise for the development of long-lasting therapeutic approaches to treat these poor prognosis AML patients.

2. Genomics underpinning transformation in AML

Myeloid malignancies are rarely heritable, usually emerging from sporadic acquired somatic mutations originating in hematopoietic progenitors [28, 29]. Overall, the majority of mutations found in the genome of leukemic cells represent the mutational history of the cell rather than pathogenically relevant mutations [30]. However, with increasing age and expansion of hematopoietic progenitors over time, cells accumulate mutations that may represent premalignant events. More than 2% of healthy individuals carry several genetic alterations characteristic of hematologic malignancies in the

genome of their hematopoietic cells, most of them associated with increased age [31]. Presence of AML-associated mutations in genes such as *IDH1*, *IDH2*, *DNMT3A*, *TET2*, *SRSF2* and *TP53* are associated with an increased likelihood of developing AML later in life. *FLT3* and *NPM1* mutations are not among the baseline mutations which have been observed, and as such, are likely later events in leukaemogenesis [32, 33]. As in many other cancer types, the process of leukemogenic evolution can take many years. This long-time period is known as the “pre-leukemic phase” and allows pre-leukemic HSCs to accumulate multiple genetic lesions, even in the absence of genomic instability [34, 35].

The transformation to AML is characterized by a two-hit model of pathogenesis which requires the association of two different classes of cooperating mutations, where class I mutations confer proliferative advantages, and class II mutations impair hematopoietic differentiation and/or induce the acquisition of self-renewal properties by HSCs [29, 36]. This process follows a specific evolutionary trajectory compounding several events, each of them generating a small cluster of new mutations, though only one or two are potentially pathogenically relevant [30, 37]. First, a HSC that already contains hundreds of random and benign mutations, acquires an initiating driver lesion conferring clonal advantages that are carried forward with this clone as it expands [30]. Early mutations acquired in this phase generally affect genes involved in epigenetic regulation (i.e. *DNMT3A*, *TET2*, *IDH1/2*, and the cohesin complex) to prevent cell differentiation and allow the pre-leukemic cell to survive long enough to accumulate further mutations [35]. Once the expanding clone acquires an additional cooperating driver lesion (e.g. *FLT3*-ITD or *KRAS*) it is fully transformed into a malignant leukemic founding clone, which will give rise to subsequent subclones [8, 30]. Over time, the coexistence of multiple ancestral and emerged clones creates the clonal heterogeneity responsible for disease progression [30, 38].

3. *FLT3*-ITD mutations confer a poor prognosis in cytogenetically normal (CN) AML

Chromosomal alterations are one of the most common characteristics of cancer [39] however, these events are only seen in 30-35% of AML patients, while 65-70% of patients display a normal karyotype [7, 8, 40]. Patients harboring ≥ 3 chromosome aberrations in the absence of t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22) and t(15;17)(q22;q21), form a separate category known as AML with a complex karyotype (CK-AML) [41]. CK-AML is a marker of prognosis, classifying patients into poor-risk groups [30, 42]. However, individual gene rearrangements and point mutations are not sufficient to cause AML alone; multiple acquired mutations must occur to convert normal HSCs into leukemic stem cells (LSCs) [36, 43]. This is particularly the case for cytogenetically normal (CN-AML) patients who were previously assigned an intermediate-risk prognosis in the MRC UK risk stratification [44].

FLT3-ITD mutations are rarely seen at diagnosis in patients harboring CK-AML [41, 45], however strikingly, they occur in 70% of patients with normal karyotype AML [46]. Presence of a *FLT3*-ITD mutation is a predictor of poor prognosis in CN-AML. *FLT3*-ITD mutations tend to co-occur with mutations in genes related to DNA methylation (*DNMT3A*, *TET2*, *IDH2*), histone methylation (*NPM1*, *MLL3*), myeloid transcription factors (*RUNX1*, *WT1*), and signaling mediators (*FLT3*-TKD) [47]. Among these, *DNMT3A* and *NPM1* are the most common [8]. Importantly, the mutations co-associated with *FLT3* have been demonstrated to hold prognostic significance. *FLT3*-ITD occurs in combination with *NPM1* mutations in 40% of CN-AML [48], conferring an intermediate risk prognosis. Conversely, *FLT3*-ITD mutations concurrent with *DNMT3A* mutations (13.3% of CN-AML) confer a very unfavorable outcome [49]. Concurrent mutations in *FLT3*, *NPM1*, and *DNMT3A* are associated with heavy disease burden, and poor treatment response, event-free survival (EFS) and OS [8, 9, 50, 51]. This is probably a bystander event, with *NPM1* not adding to the prognosis in the presence of the other two driver mutations. Co-occurrence of *MLL*-partial tandem duplication (*MLL*-PTD) with *FLT3*-ITD also confers a poor prognosis (25% of CN-AML) [52, 53]. The presence of mutations affecting genes encoding epigenetic modifiers such as *DNMT3A*, *IDH1/2* and *TET2* possibly induces genomic instability responsible for the induction of *FLT3*-ITD mutations, converting leukemic cells into resistant clones able to undergo clonal expansion [54].

Patients with FLT3-TKD represent approximately 5-7 % of all AML cases, and 11% of CN-AML [12, 55]. FLT3-TKD mutations again occur most frequently with NPM1 mutations (10-15%) [48], but unlike FLT3-ITD mutations, are not consistently associated with inferior survival [52, 56]. However, in concert with MLL-PTD, FLT3-TKD confers a significantly poor prognosis [9]. The emergence of additional TKD mutations in FLT3-ITD+ AML patients (FLT3-ITD-TKD) represents an important mechanism of resistance following use of TKIs [16, 19, 57-59], leading to a very poor prognosis. Following the same pattern of co-occurrence as single mutants, dual mutant FLT3 tends to co-occur with DNMT3A and NPM1, and is negatively associated with TP53 and NRAS mutations [8, 9]. Functional characterization of mutant FLT3 and the modifying effect of co-operative mutations will be crucial for the identification of improved treatment strategies for each molecular subtype of AML.

4. Signaling pathways regulated in FLT3 mutant AML

FMS-like Tyrosine Kinase 3 (FLT3; also known as CD135) is a membrane-bound receptor tyrosine kinase (RTK) which localizes to the cell surface following post-translational glycosylation [60]. FLT3 belongs to the type III RTK family; containing five immunoglobulin-like extracellular domains, a transmembrane domain, and intracellular juxtamembranous and split kinase domains (Figure 1). All five members of the type III RTK subclass (KIT, PGDFR α , PDGFR β , CSFR, and FLT3) have critical roles in normal hematopoiesis regulating proliferation, differentiation and survival [61].

FLT3 is activated by its endogenous ligand (FLT3 ligand; FLT3L) in an autocrine and paracrine manner, in synergy with interleukin-3 (IL-3), stem cell factor, or other cytokines [60] (Figure 1). Binding of FLT3L leads to dimerization of the FLT3 receptor, and a conformational change in the intracellular tyrosine kinase domains, exposing phosphoryl acceptor sites [62, 63]. Subsequent trans-autophosphorylation of FLT3 leads to binding of adaptor proteins such as SHP2, Grb2, and Src family kinases [64-66] inducing activation of downstream kinase signaling pathways including MAPK [67], STAT [68], and PI3K [69], driving cell growth and survival.

FLT3 is predominantly expressed by hematopoietic progenitor cells [70], but expression is lost or reduced during differentiation into mature lymphoid and myeloid cells [71-73]. The majority of AMLs express the FLT3 receptor and its ligand [74]. FLT3-ITD and FLT3-TKD mutations disrupt the auto-inhibitory resting conformational state of the receptor, inducing constitutive activation of FLT3 and ligand-independent cell growth [12, 75, 76]. The expression of the FLT3-ITD mutation in mouse models is sufficient for the development of fatal myeloproliferative neoplasm [77-79], although an extra genetic “hit” is required for the development of acute leukemia [80-82]. While not as well characterized, FLT3-TKD mutations also induce myeloproliferative disease in mice, but with a less aggressive phenotype compared to FLT3-ITD carriers [83, 84].

The cellular pathways differentially activated by FLT3-ITD and FLT3-TKD mutants in comparison to wild-type FLT3 have been largely characterized in mouse models, and in relation to canonical FLT3 proliferation and survival signaling pathways. FLT3-ITD and FLT3-TKD forms lead to activation of similar pathways to the wild-type ligand-stimulated receptor, with some divergences (Figure 2). Both FLT3-ITD and FLT3-TKD mutants display increased retention in the endoplasmic reticulum, from where they display differential phosphorylation patterns and kinase activity [85, 86], which may contribute to divergent signaling. FLT3-TKD is associated with increased activation of SHP1 and SHP2 phosphatases [87]; SHP1 in particular is known to negatively regulate JAK signaling [88]. Consistently, FLT3-TKD mutants display low levels of JAK2 and STAT3 activation (Figure 2). In contrast, FLT3-ITD mutants are associated with a high level of JAK/STAT signaling (Figure 2), including phosphorylation of STAT5A; which is not induced by the wild-type receptor [89]. This suggests that increased JAK/STAT signaling is at least one mechanism by which FLT3-ITD cells may confer an oncogenic advantage over FLT3-TKD mutant activated cells; indeed JAK inhibitors have been shown to elicit synergistic cell death in combination with FLT3 inhibition in FLT3-ITD AML cells *in vitro* [90]. Additionally, FLT3-ITD expression is associated with decreased protein expression of transcription factors Pu.1 and C/EBP α [89], which may contribute to impaired cell differentiation.

Currently, there is a paucity of studies characterizing signaling pathways activated downstream of FLT3-ITD-TKD dual mutations. FLT3-ITD-D835Y retains the ability to activate STAT5A [89], and

is also associated with increased expression of DNA repair protein RAD51 and anti-apoptotic protein BCLxL, which may contribute to drug resistance and enhanced cell survival [57]. Further proteome-wide studies characterizing mutant FLT3 signaling in human samples are warranted.

5. Relapse and resistance is common in FLT3-ITD AML

The cornerstone of therapy for AML at diagnosis is induction therapy with cytarabine for 7 days and an anthracycline antibiotic daunorubicin or idarubicin for 3 days (popularly known as “7 + 3”), and achieves remission rates of 63% [91, 92]. This is followed by additional intensive consolidation chemotherapy [93-95]. For those that achieve complete remission (CR), allogeneic hematopoietic stem cell transplant (alloH SCT) leads to the highest probability of long-term survival [96].

Although more than half of all AML patients achieve initial CR, the majority will relapse [97, 98], with expected survival for relapsed patients approximately 10% [99]. The poor outcomes following relapse are dictated mostly by the biological heterogeneity of the disease, which includes a series of patient and disease-associated factors. Refractory disease and relapse usually results from the clonal evolution of leukemic cells which gives rise to biologically distinct, and increasingly drug resistant, malignant populations [4, 7]. Cytotoxic chemotherapies induce DNA damage, thus promoting the acquisition of additional mutations which may alter cell growth and drug sensitivity [7, 47, 100, 101]. Furthermore, chemotherapy itself contributes in the selection of pre-existing and treatment-induced drug resistant clones that will outgrow and drive relapse.

Patients relapsing early after achieving CR (<1 year) most likely relapse due to a chemotherapy resistant disease, whereas those that relapse later show evidence of chemo-refractory, quiescent leukemia-inducing cells, also referred to as leukemic stem cells (LSC; CD34⁺/CD38⁻/CD123⁺ cells) [102, 103]. Patients presenting with higher proportions of LSC demonstrate significantly lower relapse free survival (RFS) than patients with less LSC [103, 104]. Like AML blast cells, LSCs require mutations in genes encoding for signaling proteins and transcription factors to promote overt AML [105, 106]. Furthermore, LSCs are more chemoresistant than AML blast cells [26] making their characterization an important research endeavor.

FLT3-ITD mutant AML patients carry CD34⁺/CD38⁻/CD123⁺ LSCs in the bone marrow that uniformly harbor ITD mutations. FLT3-ITD mutant LSC are also common in patients diagnosed as wildtype FLT3, who then relapse with FLT-ITD mutant AML. The presence of FLT3-ITD in LSCs is associated with expression of CD123 [107, 108], encoding for the IL3 receptor subunit alpha (IL-3RA) which is overexpressed in 45% of AML patients. FLT3-TKD mutations, on the contrary, are not associated with high fractions of CD34⁺/CD38⁻ cells [107]. The majority of FLT3-ITD⁺ AML patients harbor LSCs which are also FLT3-ITD⁺. This supports the notion that FLT3-ITD is a driving event in leukemogenesis, and may contribute to the high propensity of relapse in FLT3-ITD⁺ AML. Presence of LSCs with FLT3-ITD mutations is associated with poor clinical outcomes [109, 110]. This justifies efforts to track LSCs, which may have clinical utility in monitoring minimal residual disease (MRD) [111].

Characterization of chemotherapeutic resistance afforded by LSCs is an important area of research particularly in the context of minimal residual disease (MRD) monitoring, however, the low abundance of LSCs and their infrequency in patients' bone marrow makes this a challenging endeavor. Instead, characterization of LSCs using AML cell line models has gone some way to revealing the intracellular enzyme activities associated with the ‘stemness’ of LSCs. LSC cell line models harboring FLT3-ITD mutations are yet to be developed. However, the Kasumi-1 AML cell line, which possesses a mutation in another type III RTK, cKIT, (CD34⁺, cKIT-N822K) is considered a valuable model for the study of LSC properties. Kasumi-1 cells show upregulated CEBPA, BMI-1 and NOTCH-1 gene expression [112], which may provide protection to these primitive cells from traditional and precision therapies through altered cellular differentiation.

Ultimately, the identification of models to study LSCs harboring FLT3-ITD mutations or complex cytogenetics will offer the best hope of characterizing the oncogenic signaling that may afford LSC specific targeting in high-risk or poor prognosis AML patients, but until appropriate models can be developed the rarity of these cells precludes unbiased proteome-wide analysis.

6. FLT3 Targeted Therapy

6.1 Tyrosine Kinase Inhibitors in clinical development for AML

One of the first TKIs developed for clinical use, the BCR-ABL inhibitor imatinib, revolutionized the therapeutic landscape for chronic myeloid leukemia (CML) patients. Since the clinical introduction of TKIs for CML therapy in 2001 [113], 10-year survival rates have improved from 20% to over 80% [114, 115]. Following this, there have been many attempts to develop TKIs to replicate this striking response in other malignancies driven by constitutive kinase activation, including the development of FLT3 TKIs for AML. However, despite initial favorable responses, the majority of clinical trials for FLT3 TKI monotherapy have seen the development of treatment resistance and relapse in less than 3 months of therapy. Combination therapeutic approaches are returning promising results, but the challenge remains to identify which patients will respond. Second generation FLT3 inhibitors offer highly potent and specific FLT3 inhibition compared to first generation FLT3 inhibitors (Figure 3), however it remains to be determined whether this translates into increased clinical benefit. Resistance to each FLT3 TKI is associated with a different profile of FLT3 mutations (Table 1). The ATP-competitive FLT3 TKIs are designated either type I or type II dependent on the mechanism of FLT3 inhibition; type I inhibitors bind the active form of the kinase that is associated with a “DFG in” (Asp-Phe-Gly – DFG motif at the N terminus of the activation loop) conformation. Type II inhibitors bind the “DFG-out” (conformation that is only accessible when the kinase is inactive (Figure 1). As FLT3 mutations affect the conformation of the receptor, the sensitivity of FLT3 mutants towards TKI varies between the different activating mutations present [116] (Table 1 and Table 2).

6.1.1 First-Generation TKIs

Midostaurin (PKC412): Midostaurin is an oral multi-targeted TKI that promotes direct and indirect inhibition of mutant FLT3 receptor phosphorylation. Midostaurin has been shown to induce cell cycle arrest and apoptosis in both FLT3-ITD and FLT3-D835Y mutant cell lines with an IC₅₀ of less than 10nM [117]. Midostaurin was also effective in a panel of FLT3-ITD-TKD mutant lines, although TKD1 mutations may confer some resistance [118] (Table 1 and Table 2). In a phase II study of midostaurin monotherapy, 14 out of 20 patients with relapsed/refractory AML or myelodysplastic syndrome with FLT3 activating mutations, achieved approximately 50% reduction in peripheral blasts [119]. In a phase IIB study of 95 patients with relapsed/refractory AML or myelodysplastic syndrome irrespective of FLT3 status, 50 mg or 100 mg midostaurin administered twice daily showed acceptable tolerability and high rates of blast reduction, with one FLT3-ITD+ patient achieving partial remission (PR) [120]. A phase III placebo controlled study followed, using induction and consolidation chemotherapy regimens combined with midostaurin and followed by one year maintenance with midostaurin in 717 patients with newly diagnosed FLT3 mutated AML (ITD or TKD) [20]. Despite no significant difference in rates of CR, patients treated with midostaurin achieved significantly longer event free survival (EFS) and median OS. This study led to the FDA approval of Midostaurin in combination with chemotherapy in newly diagnosed AML patients with mutated FLT3, providing the first hope for realization of a precision therapy in AML.

Sorafenib (BAY 43-9006): Sorafenib is an oral multi-targeted TKI, FDA approved for treatment of solid malignancies including; renal cell carcinoma, hepatocellular carcinoma and thyroid cancer [121]. Sorafenib is an inhibitor of FLT3-ITD, but is not active against many FLT3-TKD1 and –TKD2 mutations (Table 1 and Table 2). A phase I trial of 16 patients reported that oral administration of sorafenib reduced blast percentage in 3 FLT3 wild-type and 6 FLT3-ITD+ patients, but no response was observed in patients carrying FLT3-D835, with or without a concurrent FLT3-ITD mutation [122]. The combination of sorafenib with clofarabine and cytarabine in pediatric relapsed/refractory acute leukemia patients showed good anti-leukemic activity and tolerability [123], with 83.3% (10/12) of patients displaying a decreased blast percentage and 6 patients (3 FLT3-ITD+, 3 FLT3 wild-type) achieving CR. In a phase I/II study, combination therapy of sorafenib with cytarabine and idarubicin in treatment naive AML patients under 65 years, 83% of FLT3 wild-type patients and 95% of FLT3

mutant patients showed a CR or PR [124]. Responders included patients with D835 and ITD-D835 mutations, demonstrating that combination therapy may sensitize D835 mutants to sorafenib treatment. In a randomized, double-blind phase II clinical trial, 267 treatment-naïve AML patients under the age of 60 were randomized to receive induction and consolidation chemotherapy combined with either placebo or sorafenib [125]. Despite no significant difference in 3-year OS rates, patients receiving sorafenib displayed a significantly increased event- and relapse-free survival. Further analysis of OS after long-term follow up is now warranted.

Sorafenib has also been assessed in combination with the hypomethylating agent azacytidine, in a phase II trial of relapsed/refractory FLT3-mutant AML [126]. Of 37 patients enrolled, 36% demonstrated a clinical response, showing promise for FLT3 inhibitor combinations beyond the highly toxic chemotherapy regimens. Additionally, unlike chemotherapy regimens, sorafenib and azacytidine combination therapy was not associated with a high level of induced FLT3 ligand expression. In a small trial of sorafenib plus azacytidine in older FLT3-ITD+ AML patients, unfit for chemotherapy, 78% achieved a clinical response [127]. Sorafenib and azacytidine combination therapy is currently in clinical trial for evaluation in 27 older AML patients who are treatment naïve, with interim results demonstrating significantly longer durations of remission in patients who received sorafenib compared to those receiving azacytidine only (14.5 months compared to 3.8 months, respectively) [128].

Sunitinib (SU11248): Sunitinib is an oral multi-targeted TKI with FDA approval for treatment of metastatic renal cell carcinoma, gastrointestinal stromal tumors, and pancreatic neuroendocrine tumors [121]. Sunitinib is effective against FLT3-ITD and a subset of FLT3-TKD mutations (Table 1), with sunitinib treatment able to overcome PKC412 resistance *in vitro* [16, 129]. In a phase I trial of 15 relapsed/refractory AML patients treated with sunitinib, patients harboring FLT3 activating mutations achieved only PR [130]. In a study of relapsed/refractory pediatric FLT3-ITD+ AML, patients were administered sorafenib combined with chemotherapy, followed by sunitinib treatment upon loss of responsiveness to sorafenib [16]. Sorafenib resistance was associated with the emergence of secondary FLT3-TKD mutations, with sunitinib demonstrating efficacy against D835H and F691L point mutations, but not D835Y (Table 2). This study demonstrates how knowledge of the resistance profile of FLT3 inhibitors combined with the characterization and monitoring of FLT3 mutations throughout patient therapy, may lead to clinical benefit through sequential TKI administration.

Lestaurtinib (CEP-701): Lestaurtinib is an oral TKI that inhibits FLT3-ITD phosphorylation with an IC₅₀ of 3 nM [131]. In a phase I/II clinical trial with 17 refractory/relapsed AML patients expressing FLT3-activating mutations, lestaurtinib monotherapy demonstrated minimal toxicity and led to a significant reduction in peripheral or bone marrow blasts in 5 patients. However, these responses lasted less than 3 months, possibly due to the advanced and pretreated nature of disease. Encouragingly, *ex vivo* drug assays demonstrated potent inhibition of FLT3 phosphorylation by lestaurtinib in samples from all patients that displayed a clinical response. Samples from 3 further patients showed reduced FLT3 activity *ex vivo*, however did not show a clinical response to lestaurtinib, suggesting reliance on alternate oncogenic signaling pathways in addition to FLT3 [132]. In a phase II trial of lestaurtinib administered as a single-agent in older patients, reduced peripheral or bone marrow blasts was seen in 60% of patients harboring FLT3-mutations and 23% of wild-type FLT3 patients. Again, clinical response correlated with drug response *ex vivo*. [133]. In a larger randomized phase II trial with FLT3-ITD+ AML patients in first relapse receiving chemotherapy alone or chemotherapy followed by lestaurtinib, there was no significant improvement in response rates or OS between both treatment groups. However, patients that demonstrated reduction in FLT3 phosphorylation to below 15% of baseline whilst receiving lestaurtinib displayed significantly better median survival than those that did not [134]. Together, these studies highlight the validity of targeting FLT3 signaling for treatment of AML, and suggest that monitoring FLT3 inhibition and *ex vivo* drug screening may help determine which FLT3 inhibitor or therapy combination to use to elicit the greatest possible patient response.

Tandutinib (MLN-518): Tandutinib is a FLT3, KIT and PDGFR TKI active against the autophosphorylation of FLT3-ITD with IC₅₀ ranging from 10-100 nM [135]. In a phase I trial with

tandutinib, 40 patients with AML or high-risk MDS were treated with doses ranging from 50 mg to 700 mg twice daily. Two out of five FLT3-ITD+ patients evaluable for assessment showed anti-leukemic activity, with a decrease in both bone marrow and peripheral blasts when treated with doses ranging from 525 mg - 700 mg. However, within two months both patients experienced disease progression [136]. The development of resistance to tandutinib follows the same pattern of most TKIs, arising from the acquisition of an additional point mutation in the kinase domain at residue D835 [137].

6.1.2 Second-Generation TKI

Quizartinib (AC220): Quizartinib is a more selective and potent FLT3 inhibitor compared to the first-generation agents (Figure 3) and therefore displays less off-target effects, however is not active against a range of FLT3-TKD mutants (Table 1 and Table 2). In a phase I study of quizartinib administered daily to 76 relapsed/refractory AML patients irrespective of FLT3 status, 53% of FLT3-ITD+ patients and 14% of FLT3 wild-type patients displayed a clinical response. [138]. A phase II study assessed the efficacy and safety of two lower doses of quizartinib monotherapy in 76 FLT3-ITD+ patients with relapsed/refractory AML [139]. Patients were randomized to receive either 30 mg or 60 mg quizartinib daily, with 50% of patients in each group achieving composite CR (defined as complete remission + complete remission with incomplete platelet recovery + complete remission with incomplete hematological recovery) [139].

A larger-scale phase II trial of 333 relapsed/refractory AML patients has recently confirmed these response rates. Two cohorts of patients were recruited; those 60 years or older, and those 18 years or older, with 56% and 46% of FLT3-ITD+ patients achieving a composite CR in each group respectively; and 36% and 30% of FLT3 wildtype patients [140]. This led to a phase III randomized controlled trial of refractory/relapsed AML, with 367 FLT3-ITD+ patients randomized to receive quizartinib or standard of care chemotherapy [141]. The use of quizartinib in this trial significantly out-performed chemotherapy, with median OS rates of 27 and 20.4 weeks, respectively. These favorable results have prompted the initiation of a phase III clinical trial evaluating quizartinib for newly diagnosed AML in combination with standard of care [142]. The safety of quizartinib plus chemotherapy has been demonstrated in a pilot study of 55 older patients with newly diagnosed AML (median age of 69 years). Among the 42 evaluable patients, 33 achieved CR; including all 4 FLT3-ITD carriers [143].

Gilteritinib (ASP2215): Gilteritinib is a dual inhibitor of FLT3 and AXL. Gilteritinib is active against both FLT3-ITD and -D835 mutations, and concurrently inhibits the AXL kinase which is associated with FLT3 inhibitor resistance [144]. In a phase I-II study of 252 relapsed/refractory AML patients, gilteritinib was well tolerated, with 37% of FLT3-ITD+ patients achieving composite CR, along with 9% of FLT3 wild-type patients [145]. Of the patients that had received previous treatment with sorafenib, 49% achieved a clinical response, and 54% of patients with dual FLT3-ITD-D835 mutations achieved a composite CR, demonstrating that gilteritinib may overcome some of the acquired resistance mechanisms observed in response to preceding FLT3 TKI treatment. Following on from these promising results, a phase III trial of gilteritinib in FLT3 mutant relapsed/refractory AML is in progress [146].

Crenolanib: Crenolanib is an oral FLT3 TKI active against FLT3-ITD and FLT3-TKD mutations, however its pharmacokinetics dictate drug administration multiple times a day. In a phase II trial of 34 FLT3 mutant patients, 47% achieved a clinical response [147]. In another small trial of 65 patients with refractory/relapsed AML [148], 50% of patients that had not received prior FLT3 TKI therapy achieved a clinical response. Of those that had prior exposure to FLT3 inhibitors, 31% demonstrated a clinical response. Interestingly, relapse following crenolanib treatment was not associated with the acquisition of further FLT3 mutations. Crenolanib combined with chemotherapy is currently in trial for treatment of newly diagnosed AML [149, 150].

7. Mechanisms of relapse

7.1 FLT3-mediated mechanisms

Chemotherapy plays a significant role in disease progression in AML, instigating new mutations in founding clones or one of their subclones, which then can undergo selection and clonal expansion [7]. Chemotherapies can also select for resistant clones or LSCs (discussed above) which are present at diagnosis at very low frequencies [151]. Patients with FLT3-ITD mutation often form resistance to TKIs following the acquisition of point mutations in the activation loop of the tyrosine kinase domain 1 (i.e. N676) or 2 (most frequently D835), or the “gatekeeping” domain (i.e. F691) in FLT3 [151] (Table 1). These additional mutations in the FLT3 receptor (Figure 1) alter the structure and activation status of the activation loop, locking the receptor in a constitutively open conformation [152]. These mechanisms were first identified in a FLT3-ITD AML patient treated with midostaurin who developed resistance following the acquisition of a single amino acid substitution at position 676 (N676K) (Figure 1 and Table 1). The substitution destabilized receptor conformation, inhibiting drug binding, and was recognized as the sole cause of resistance to midostaurin [153]. Whether this additional lesion was acquired or already present in subclones selected under treatment remains to be clearly determined, but provides a biochemical mechanism for resistance to TKIs that is now under intensive investigation. Knowledge of the sensitivities for each TKI against recurring FLT3 mutations is beginning to provide us with a road map for selection of the next line of therapies (Table 1 and Table 2). For example, if a FLT3-ITD AML patients develops resistance to sorafenib due to the selection of a subclone harboring a FLT3-ITD-D835 mutation, or this mutation is induced following the use of chemotherapies, the patient is still likely to be sensitive to midostaurin (Table 2). So TKI substitution in real-time could be effective strategy. Further profiling of specific mutations and their sensitivities to TKIs are needed to obtain a comprehensive understanding of which mutations are sensitive to which TKIs or which inhibitors are best for each mutation (Table 2).

FLT3 inhibition using small molecules is an effective treatment strategy for FLT3-ITD AML; however, future drug development may see new TKIs that target alternative-binding sites in FLT3 that see them to remain active even in the presence of addition FLT3 mutations. It is also interesting that in most cases of resistance, the resistance mechanism preserves FLT3 signaling. Therefore, the rational design of combinations of therapies to provide therapeutic inhibition of FLT3 signalling and also overcome the development of resistance is an important research endeavour and remains the focus of research efforts worldwide.

7.2 Non FLT3-mediated mechanisms

The use of TKIs in FLT3-ITD mutant AML patients leads to the clearance of circulating blasts, however, this treatment strategy has little efficacy for blasts protected within the bone marrow niche. It is now clear that the bone marrow stroma enhances survival mechanisms by the production of signals that activate cooperative oncogenic signaling. Patients resistant to quizartinib maintain RAS/MAPK signaling downstream of FGFR1 through the increased expression of FGF2 in the bone marrow stromal cells [154]. The combination of a hypomethylating agent such as azacytidine or decitabine with quizartinib or sorafenib, ameliorates the protection afforded by the bone marrow, inducing *in vivo* and *ex vivo* apoptosis, growth inhibition, and terminal differentiation in FLT3-ITD AML blasts [155].

Constitutive activation of FLT3 signalling through means other than mutations to the FLT3 receptor itself may also contribute to TKI resistance. Acquisition of mutations in FLT3 downstream signalling mediators, such as NRAS [156, 157], may enable conservation of FLT3 signalling in the presence of FLT3 inhibitors, leading to relapse.

The contribution of elevated expression of FLT3L is also an important mechanism of resistance to TKIs. FLT3L can originate in the bone marrow stroma and T lymphocytes *in vivo* following chemotherapy and impairs the efficacy of TKIs [158]. ERK signaling mediates the protective effect afforded by FLT3L with MEK inhibition now an important drug target [158]. Combinations of drugs that target mutant FLT3 and bone marrow stroma signalling are important strategies that require further investigation. It is important that these studies include analysis of cooperative and alternative signaling pathways both pre- and post-treatment to help in the prediction of which pathways may contribute to the resistance that may subsequently form. Work in this regard will help us to stratify

patient treatment based on signaling pathway signatures rather than selecting therapies purely based on genetics.

8. Conclusions

Recent landmark genomics studies have laid the foundations for the development of treatment strategies tailored to individual patients. These sophisticated studies reveal several driver mutations that are now well linked to disease development, progression, and relapse in AML. This is indeed the case for the most commonly mutated gene in AML – FLT3. Advancements in genomics technologies coupled with access to archival AML tissue of thousands of well-defined patients, has facilitated the unequivocal association between activating mutations in FLT3 and a poor prognosis, and high rate of relapse [9].

Since the uncovering of activating mutations to the *FLT3* gene [11] and the subsequent realization of the high frequency of these events [159, 160], rapid development of broad and more recently, selective inhibitors of FLT3, have flooded the AML clinical trials space. Both classes of inhibitors induce an initial favorable clinical response, however the development of inhibitor resistance, followed by rapid disease relapse occurs in almost every case. Relapse commonly develops following the acquisition of a secondary, drug resistant lesion to the *FLT3* gene, with resistance mechanisms now identified for almost all of the FLT3 TKIs in clinical use (Table 2).

The important correlation between the level of on-target inhibition (in this case, phosphorylation of FLT3) of some TKIs, and the positive clinical response seen, provides us with an opportunity to monitor treatment for patients' blasts in near real-time [25]. Furthermore, sophisticated and quantitative unbiased phosphoproteomic profiling [24] now affords us with the tools necessary to uncover and monitor the key signaling pathway divergences associated with each specific FLT3 mutation, such that the design of future therapeutic approaches may hinge on the identification of activated signaling pathways rather than on somatic mutations alone. These tools are beginning to provide us with the means to position therapies for precisely the right patient, and to predict the level of treatment efficacy and durability. However, more work is necessary to link recurrent driver mutations and cooperating passenger lesions with their respective oncogenic signaling pathways, particularly for mutations emerging at relapse (Figure 2, Figure 3, Table 1 and Table 2).

FLT3 inhibitor monotherapy is insufficient to achieved sustained clinical responses. This is likely due to more than one driver gene mutation is required for the development of AML. Therefore, it follows that successful AML therapy will require multifactorial gene targeting. In 2017, the FDA approved the use of four new drugs for the treatment of AML, providing hope that clinical teams will have the armory to prescribe the right combination of drugs for the right patient [161]. It is further hoped that research teams will collaborate with clinical teams to monitor on-target efficacy and determine treatment response in real-time to allow for bidirectional communication between clinicians and scientists so that dynamic drug exchange can occur before disease burden becomes too high. Among the newly FDA approved drugs is the multikinase inhibitor Midostaurin, approved for administration in combination with chemotherapy in front-line treatment of FLT3 mutant AML. This development occurred on the back of the results of the RATIFY trial, which demonstrated a significant improvement in median survival for patients on Midostaurin in combination with chemotherapy (74.7 months compared to 25.6 months for chemotherapy alone) [20]. This supports the notion that combination therapies may be required to achieve sustained therapeutic benefit. The toxicity of the 7+3 AML chemotherapy regimen, precludes this as a treatment option for some patients, particularly the elderly. Further combinations of FLT3 inhibitors with hypomethylating agents which show a lower toxicity profile, are showing promise in clinical trial [128] and need to be further investigated.

A good proportion of FLT3 wild-type patients display response to FLT3 inhibitors, suggesting that genomic profiling alone may not provide optimal patient stratification, however, little data is currently available on the role that these combinations play in controlling oncogenic signaling in

wildtype FLT3 AML. *Ex vivo* profiling of drug response may aid in identification of the most effective drug combination for each individual patient, and may also help to identify effective secondary therapies in the resistance setting [16, 131-133]. However, detailed investigations into the key signaling pathway divergences linked to FLT3 mutations will hopefully provide us with a means to position therapies precisely for the right FLT3 patient subtypes (Figure 2), with future work in this space likely to uncover novel therapeutic treatment targets that will improve combinatorial targeting of mutant FLT3 in AML.

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9. Abbreviations

aa	Amino acid
alloHSCT	Allogeneic hematopoietic stem cell transplant
AML	Acute myeloid leukemia
CK-AML	Complex karyotype AML
CML	Chronic myeloid leukemia
CN	Cytogenetically normal
CR	Complete remission
Del	Deletion
DFS	Disease-free survival
EFS	Event-free survival
FLT3	FMS-like tyrosine kinase 3
FLT3L	FLT3 ligand
FLT3-mut	FLT3 mutation
HSC	Hematopoietic stem/progenitor cell
IL-3	Interleukin-3
Indels	Small insertions or deletions
ITD	Internal tandem duplication
LI-C	Leukemia-inducing cells
LSCs	Leukemic stem cells
MDS	Myelodysplastic syndrome
MLL-PTD	MLL-partial tandem duplication
MRD	Minimal residual disease
OS	Overall survival
PDGF	Platelet-derived growth factor
PR	Partial remission
RFS	Relapse-free survival
RTK	Receptor tyrosine kinase
TK	Tyrosine kinase
TKD	Tyrosine kinase domain
TKIs	Tyrosine kinase inhibitors

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Tables

Table 1. Mutations associated with resistance to FLT3 inhibitors.

FLT3 Inhibitor	Class, Type	Mutation/amino acid change	Tyrosine Kinase Domain
PKC412 (Midostaurin)	First generation; Type 1	N676K [153]	TKD1
		672E [162]	
		N676D [163, 164]	
		F691L, G697R [163]	
		N676I, N676S, F691I [164]	
Sunitinib	First generation; Type 1	A627P, F691L [18]	TKD1
		D835Y [16]	TKD2
		Y842C [18]	
Lestaurtinib	First generation; Type 1	A627P [18]	TKD1
Sorafenib	First generation; Type 2	F691L [16, 18, 164]	TKD1
		A627P [18]	
		D835H [16, 165]	TKD2
		D835V/F/I/del/Y/A [165]	
		F691I, Y842N [164]	
		Y842C [18]	
Tandutinib	First generation; Type 2	D835Y [137]	TKD2
Quizartinib	Second generation; Type 2	F691L [16, 18, 58]	TKD1
		A627P [18]	
		D835V/F/I/del/Y [16, 58, 165]	TKD2
		D835H [16]	
		Y842C/H [58]	
Gilteritinib	Second generation, Type 1	F961L [157, 166]	TKD1
Crenolanib	Second generation, Type 1	F691L [167]	TKD1

Table 2. Mutation-specific response to FLT3 inhibitors: Fold change in IC50 compared to FLT3-ITD. References are located in Supplementary Information, References S1.

FLT3-mut	Midostaurin	Sorafenib	Sunitinib	Lestaurtinib	Tandutinib	Quizartinib	Crenolanib	Gilteritinib
FLT3-ITD	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D835Y	-1.06	108.11	18.52	1.14	18.18	83.33	1.02	-1.14
D835V	-3.70	-	13.75	-	18.18	19.11	-	-2.27
ITD-D835Y	6.00	1678.00	7.22	-1.11	-	182.00	1.00	-
ITD-D835V	8.00	2602.00	3.25	-	-	560.00	1.00	-

Yellow indicates mutations show no difference in sensitivity to TKIs compared to ITD mutations. Green shades indicate that mutations are more sensitive to TKI than ITD mutations. Red shades indicate mutations are more resistant to TKIs than ITD mutations.

Figure Captions

Figure 1: Domains and AML-associated mutations of the FLT3 receptor tyrosine kinase. The FLT3 kinase is a 993 amino acid protein comprised of extracellular, transmembrane, juxtamembrane, and split kinase domains. Mutations which have been reported in AML are notated on the right hand side of the figure. References are located in Supplementary Information, References S1.

Figure 2: Canonical FLT3 and mutant FLT3 signaling pathways. Similar and divergent signaling pathways of wildtype and mutant FLT3 identified by functional genomic studies and large scale proteomic profiling experiments. **A)** Upon binding of the FLT3 ligand (FLT3L), the FLT3 receptor undergoes a change in conformation and trans-autophosphorylation. **B)** FLT3-ITD, **C)** FLT3-TKD and **D)** FLT3-ITD-TKD dual mutations lead to constitutive, ligand independent activation of the FLT3 receptor. Subsequent recruitment of adaptor proteins effects the activation of downstream kinase signaling pathways, such as MAPK, JAK-STAT, and PI3K. Coloring indicates activation of pathway phosphorylation or expression changes associated with the wildtype and mutant FLT3 receptor forms. Numbers indicate amino acid residues of the human protein sequence. References are located in Supplementary Information, References S1.

Figure 3: Kinase targets of first- and second- generation FLT3 inhibitors. Kinase trees depicting the dissociation constant (K_d , (nM)) of the target kinases of each inhibitor. Size of red bubble is proportional to the K_d value, with a larger bubble indicating a lower K_d . First generation inhibitors = 1st, second generation inhibitors = 2nd. Brackets indicate whether the inhibitor is type I or type II. Illustrations reproduced court References are located in Supplementary Information Reference S1.esy of Cell Signaling Technology, Inc. (www.cellsignal.com) and created using KinMap. References are located in Supplementary Information, References S1.

Figures

Figure 1:

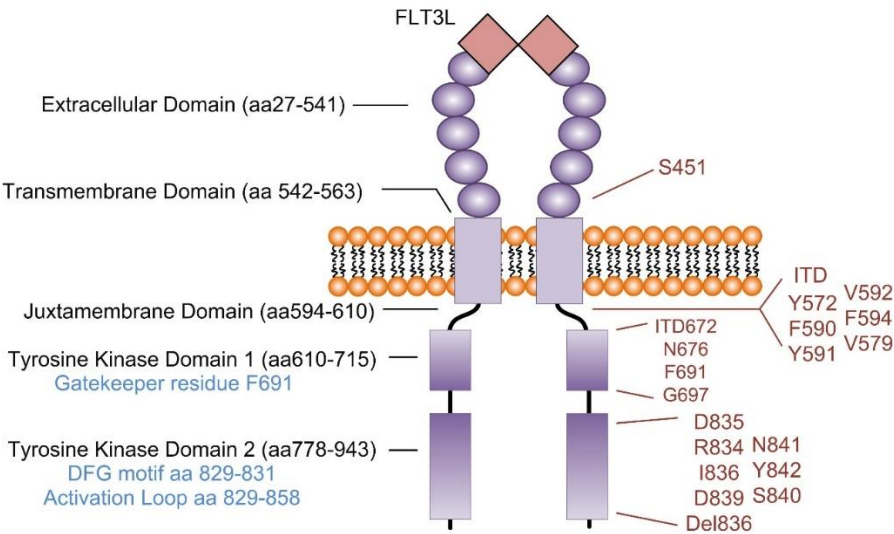


Figure 2:

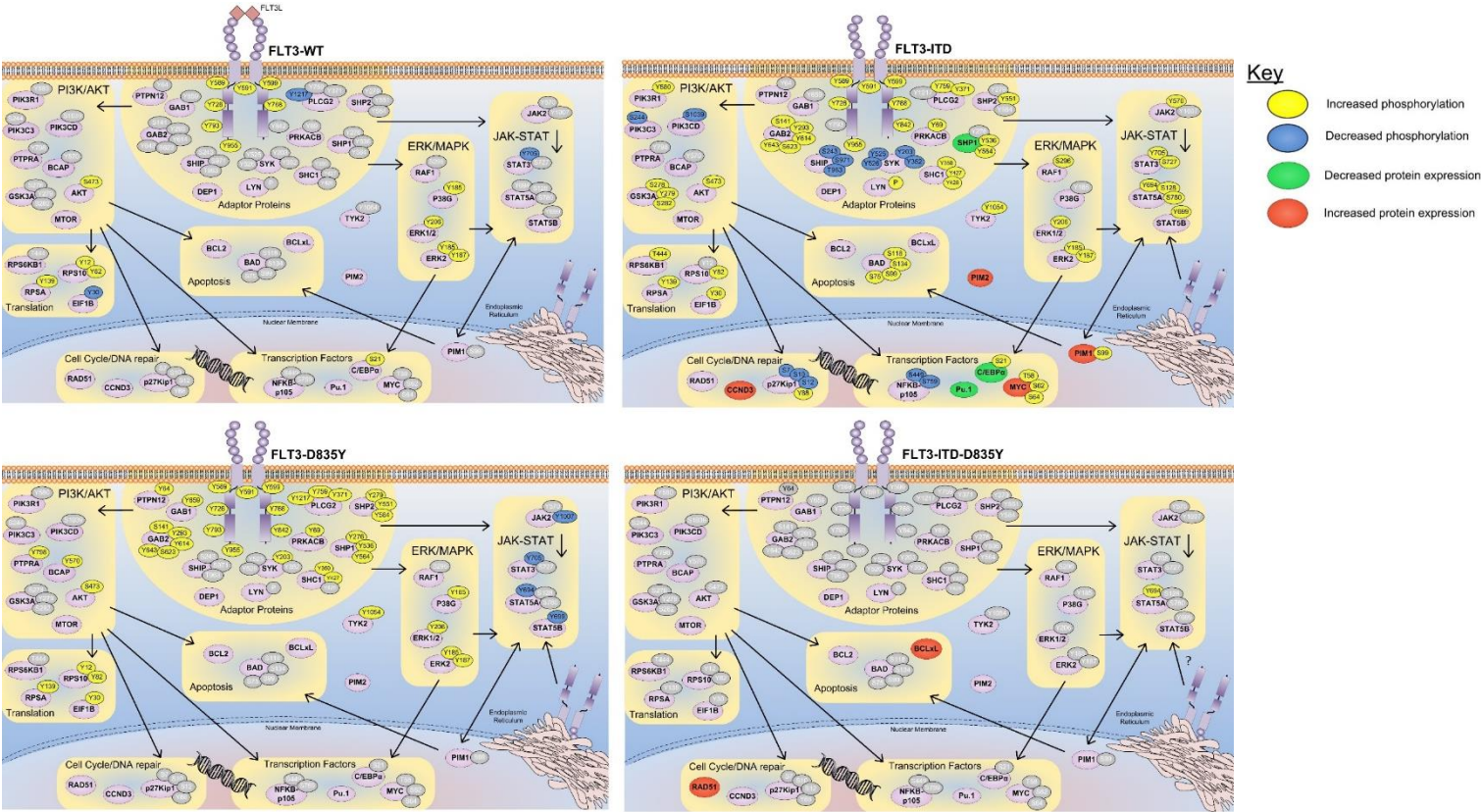


Figure 3:

