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Marion Dubuissez
Centre de recherche de l'Hôpital Maisonneuve-Rosemont
Djazia Haferssas
Centre de recherche de l'Hôpital Maisonneuve-Rosemont
Jonatan Barrera-Chimal
Centre de recherche de l'Hôpital Maisonneuve-Rosemont
Clémence Messmer
Centre de recherche de l'Hôpital Maisonneuve-Rosemont
El Bachir Affar
Maisonneuve-Rosemont Hospital Research Center and Department of Medicine, University of Montréal
https://orcid.org/0000-0002-6374-3683
Bruno Larrivée
Maisonneuve-Rosemont Hospital Research Center and Department of Medicine, University of Montréal
Xue-Song Liu
ShanghaiTech University https://orcid.org/0000-0002-7736-0077
Casimiro Gerarduzzi (casimiro.gerarduzzi@umontreal.ca)
Centre de recherche de l'H&#x00F4 https://orcid.org/0000-0001-7098-1455

Article

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Marion Dubuissez[1,2,3*], Djazia Haferssas[1,3*], Jonatan Barrera-Chimal[3], Clémence Messmer[3,5], El Bachir Affar[3,5], Bruno Larrivée[1,4], Xue-Song Liu[6] and Casimiro Gerarduzzi[1,3,4]

[1] Département de pharmacologie et physiologie, Faculté de médecine, Université de Montréal, Québec, Canada

[2] Département de Microbiologie et Immunologie, Faculté de médecine, Université de Montréal, Québec, Canada

[3] Centre de Recherche de l'Hôpital Maisonneuve-Rosemont, Faculté de médecine, centre affilié à l'Université de Montréal, Québec, Canada

[4] Département de médecine, Faculté de médecine, Université de Montréal, Québec, Canada

[5]Département de Biochimie, Faculté de médecine, Université de Montréal, Québec, Canada

[6] School of Life Science and Technology, ShanghaiTech University, Shanghai, China.

*Contributed Equally

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Running Title: FLT4 promotes p53 inactivation in Pediatric Leukemia

***Corresponding author:**

Casimiro Gerarduzzi
Division of Nephrology, Maisonneuve-Rosemont Hospital CIUSSS de l'Est-de-l'Île-de-Montréal
5415, boul. de l'Assomption
Montréal, QC, Canada
H1T 2M4
Tel: 514-252-3400 ext:2813 casimiro.gerarduzzi@umontreal.ca

ABSTRACT

Aberrant Receptor Tyrosine Kinase (RTK) signaling allows cancer cells to modulate survival, proliferation and death, leading to tumorigenesis and chemoresistance. In leukemia, the RTK FMS-Related Tyrosine Kinase 4 (FLT4) (also known as VEGFR-3, Vascular Endothelial Growth Factor- 3) is deregulated and correlates with cancer progression. However, the underlying consequences of its deregulation remain to be determined. Moreover, chemotherapy treatment requires that cancer cells retain a wild type p53 (wt) in order to respond to DNA damage by tumor suppressing activities, i.e. apoptosis. p53 activity is predominantly limited by its two major negative regulators, MDM2 and MDMX, which inactivate p53 by promoting its degradation and/or cytoplasmic localization. In this study, we have shown that activation of FLT4 by either overexpression or binding of its ligand, VEGF-C, leads to an increase in MDM2/MDMX stability, inactivation of p53 and resistance to DNA damaging therapies. Through immunoprecipitation and mass spectrometry analysis, we observed that FLT4 induced phosphorylation of MDMX at Ser-314, a consensus sequence of CDK4/6. Our data revealed that phosphorylation of MDMX on Ser-314 increases the stability of MDMX, which subsequently affects MDM2 and p53 degradation and could be reversed by the CDK4/6 inhibitor Palbociclib. More importantly, leukemic cells treated with Palbociclib were more susceptible to DNA damaging induction of apoptosis and had reduced cell proliferation. Altogether, our research proposes an innovative way to reactivate p53 in pediatric leukemia through the pharmacological inhibition of FLT4 signaling, which could serve as a potential treatment option for this disease.

ABBREVIATIONS: AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CDK4/6, Cyclin Dependant Kinase 4/6; DLBC, Diffuse Large B Cell lymphoma; FLT4, FMS-Related Tyrosine Kinase 4; GFP, green fluorescent protein; IP, immunoprecipitation; MDM2, mouse double minute 2 homolog; MDMX, mouse double minute 4 homolog; MS/MS, mass

spectrometry; RTK, receptor tyrosine kinase; VEGF-C, vascular endothelial growth factor- C; VEGFR-3, vascular endothelial growth factor receptor-3; WB, Western Blot.

INTRODUCTION

Acute leukemia is a group of malignant disorders characterized by aberrant proliferation of hematopoietic stem cells and progenitors in the blood and bone marrow.[1] It is categorized into Acute Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL) according to the predominant type of cell involved. In adults, AML accounts for about 90% of all acute leukemias.[2] In contrast, ALL is the most common cancer in children.[3] In the pediatric population, chemotherapy treatments have achieved up to 90% remission.[3,4] However, the survival remains poor for 10% of these patients due to chemotherapy resistance.[5,6] Furthermore, effective doses can cause severe late effects of toxicity.[7–9] Therefore, a better understanding of these DNA-damaging therapies would permit precise treatments for leukemic cell death and lower dosages of effective treatments for reduced toxicity.

Most chemotherapies used in leukemia are DNA-damaging therapies[10] and therefore depend on activating the tumor suppressor p53 to induce tumor cell death. p53 was the first tumor suppressor to be identified and has been called the guardian of the genome due to its central role in preventing tumorigenesis. Indeed, p53 has multiple biological functions associated with tumor suppression, such as cell growth regulation, cell cycle arrest, DNA repair and apoptosis.[11,12]

The power of p53 in stress response is emphasized by its continual expression and degradation so that at any moment, p53 can be stabilized and positioned for its activity when needed. To avoid p53 lethality, two major negative regulators control its expression and activity, MDM2 (mouse double minute protein 2) and its homologous MDMX (mouse double minute 4 homolog, also known as MDM4).[13] MDM2 and MDMX regulate p53 activity by two

mechanisms.[14] First, the complex MDM2/MDMX binds to the transactivation domain of p53 and prevents its activity as a transcription factor. Second, MDM2, unlike MDMX, has an E3 ubiquitin ligase activity that can ubiquitinate p53 to be targeted for degradation by proteasomes. Therefore, p53 has a short half-life in normal conditions and is constantly expressed and degraded to be maintained at low levels. In response to stress signals such as DNA damage, p53 rapidly uncouples from the MDM2/MDMX complex allowing its stabilization and activation of tumor suppressing genes.[14]

Due to its essential role in preventing tumorigenesis, p53 is the most frequently mutated gene in human cancers, allowing tumor cell proliferation. Over 50% of solid tumors have p53 mutations, most occurring in the DNA binding domain (DBD), altering its transcriptional activity.[15] However, the frequency of p53 mutations in hematological malignancies is lower. For instance, in pediatric leukemia, p53 mutations occur in less than 10% of patients and are associated with poor prognosis.[16–19] Thus, inactivation rather than mutation of p53 likely plays a role in leukemogenesis, and reactivation of the p53 pathway represents a potential target for improved treatment. Nevertheless, the precise mechanisms responsible for preventing wild type p53 function in leukemia remain unclear, indicating the potential involvement of inhibitory mechanisms upstream. Many cancers, including leukemia, overexpress MDM2 and MDMX.[20–27] In this context, the inhibition of MDM2/MDMX represents an attractive strategy to restore p53 functions.[20] Indeed, kinases activated by DNA damage inhibit MDM2 and MDMX, leading to p53 stabilization and activation.[28,29] Whether kinases involved in oncogenic signaling also target MDM2/MDMX for p53 regulation is still unclear.

Deregulating signaling pathways, including Receptor Tyrosine Kinase (RTK), is a hallmark of cancer.[29,30] Aberrant activation from RTKs allows cancer cells to abnormally control the cellular processes of survival, proliferation and cell death, leading to tumorigenesis and drug resistance.[31] Targeting aberrantly active RTKs in leukemia could therefore provide a

cotreatment approach to chemo/radiotherapy. Several findings have supported the role of the RTK FMS-Related Tyrosine Kinase 4 (FLT4, also called VEGFR-3 (Vascular Endothelial Growth Factor- 3)) and its ligand, VEGF-C, in cancer progression.[32,33] The VEGF family plays an essential role in angiogenesis during normal hematopoiesis.[34] Several findings have supported a role of the RTK FLT4 and its ligand, VEGF-C, in AML and ALL.[30–32] While FLT4 is not normally expressed in the bone marrow of healthy donors, both FLT4 and VEGF-C are expressed in more than one third of AMLs.[30] Specifically, AML cells from 96% of pediatric patients expressed detectable VEGF-C mRNA, while 49% expressed FLT4.[33] In a separate study, VEGF-C expression was associated with an adverse prognosis in pediatric and adult AML[34] In pediatric ALL, VEGF-C protein was detected in 27% of patients, which was significantly associated with ALL treatment failure.[32] VEGF-C signaling has also been shown to play an important role in decreased drug response and chemotherapy resistance in acute leukemia.[33–35] Furthermore, *in vitro* VEGF-C treatment increased leukemic cell survival and proliferation[34,35], and protected against apoptosis.[34,35] Although there is a strong association between FLT4 and leukemia, they have been understudied, and consequently, the mechanism by which FLT4 may be implicated in perpetuating leukemia and developing resistance to therapy is unknown.

Given that ALL patients have a wild type p53 and often have elevated levels of FLT4/VEGF-C, and resistance to DNA damage- induced apoptosis, we investigated whether the modulation of MDM2/MDMX complex sits at the interface of FLT4 and p53 activity. The present study shows that FLT4 leads to increased MDM2/MDMX complex stability, potentially through CDK4/6 and eventual p53 inactivation. We have also shown that reactivating p53 in FLT4-treated leukemic cells through CDK4/6 inhibition results in their responsiveness to genotoxic drugs promoting their apoptosis and thus providing a mechanism of combination therapy.

MATERIAL AND METHODS

Cell culture

The Human Embryonic Kidney cell line (HEK293T) and the Human Bone Osteosarcoma Epithelial cell line (U2OS) from American Type Culture Collection (ATCC) were seeded in tissue culture dishes and cultured until confluence in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% of Foetal Bovine Serum (FBS, Gibco) and Penicillin/Streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

The human precursor B-ALL cell line (REH) from ATCC was seeded in 25 mL flasks and cultured until confluence in Roswell Park Memorial Institute media (RPMI 1640, Gibco) supplemented with 10% of FBS at 37°C in a humidified atmosphere of 5% CO_2.

Transient Transfections and Transductions

Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The expression plasmids pCMV-Myc-MDM2, pCMV-MDM2 C464A mutant and pcDNA3-FLT4 were purchased from Addgene (#16441, #12086 and #119230 respectively). The expression plasmid pcDNA3-Flag-MDMX was previously described.[36] The expression plasmid pcDNA3-Flag-MDMX S314A mutant was generated using Quick Change II XL site-directed mutagenesis kit (Agilent Technologies) by two steps of site-directed PCR mutagenesis with the following primers: 5'GTACTGAATGCAAGAAATTTAACGCTCCAAGCAAGAGGTACTG3' and 3'CAGTACCTCTTGCTTGGAGCGTTAAATTTCTTGCATTCAGTAC5', according to the manufacturer's instructions.

The transduction was performed using lentivirus. First, HEK293T cells were seeded at 50% of confluency. 24h after, cells were transfected using lipofectamine 2000 according to the manufacturer's instructions with plasmids for lentivirus production (3,74 µg psPAX2 + 1.25 µg

pMD2.G) and 6 μg of lentiviral vectors pHAGE-GFP (control) or pHAGE-GFP-FLT4, purchased from Addgene (#12260, #12259 #106281 and #116745 respectively). After 48h of transfection, the supernatant containing the viral particles was collected and filtered using a 0.45 μm PVDF filter. For the transduction, REH cells were plated at $1x10^6$ cells/ mL in 2 ml of a completed medium, and 2 mL of filtrated supernatant supplemented with 4-8 μg/mL of Polybrene (Sigma- Aldrich) was added to the cells. REH cells were centrifuged at 800 x g for 30 min at 32°C. 48h after transduction, GFP-positive cells from each condition were sorted by Flow cytometry (BD ARIA III) and maintained in culture in completed media.

Colony Forming Assay of REH cells

For each condition, 500 REH cells were resuspended in completed media and seeded in 1mL of 1.2% methylcellulose (Stem Cell, #4230). Cells were plated in four replicates onto 35 mm^2 tissue culture dishes and incubated in a humidified atmosphere at 37°C and 5% CO_2. After two weeks of culture, colonies consisting of at least 50 cells were counted using an inverted microscope.

Growth Curve Analysis of REH cells

For each condition, 100.000 REH cells were seeded in 6-well plate in 3 mL of complete media and treated or not with 1 μM of CDK4/6 inhibitor (Palbociclib, Selleckchem). Cells were counted every 24h for five consecutive days.

FLT4 activation with VEGF-C

For each condition, a total of $1x10^6$ REH cells were starved in 6-well plate in 3mL of starvation media overnight. The cells were treated with 100 ng/ mL of Recombinant Human VEGF-C (R&D Systems) or vehicle (H_2O, control) at different time points. After the treatment, cells were harvested at 4°C, and the protein lysates were quantified and analyzed by Western Blot.

FLT4 inhibition with MAZ51

For each condition, a total of $1x10^6$ REH cells were seeded in 6-well plate in 3 mL of starvation media a day before the treatment. The cells were then treated with 5 μM of FLT4 inhibitor (MAZ51, Sigma Aldrich) at different time points. After the treatment, cells were harvested at 4°C, and the protein lysates were quantified and analyzed by Western Blot.

Genotoxic treatment

For each condition, a total of $1x10^6$ REH cells were seeded in 6-well plate in 3 mL of completed media a day before the treatment. The cells were treated with either 0.25 μM of Etoposide (Sigma- Aldrich) or 50 nM Doxorubicin (Sigma-Aldrich) at different time points. After the treatment, cells were harvested at 4°C, and the protein lysates were quantified and analyzed by Western Blot.

FLT4 downstream signaling inhibitors

For each condition, a total of $1x10^6$ REH cells were starved in 3 mL of starvation media in 6-well plate a day before the treatment. The cells were pre-treated with 2.5 μM of CDK4/6 inhibitor (Palbociclib, Selleckchem) for 24h. After the treatment, cells were harvested at 4°C, and the protein lysates were quantified and analyzed by Western Blot.

Whole-cell extracts preparation and Western Blot

Total protein extracts were prepared from cells lysed by RIPA buffer (Pierce) containing a cocktail of protease and phosphatase inhibitors (Roche). The cell lysate was sonicated and centrifuged at 13.000 x g at 4°C. Proteins were quantified using Pierce BCA protein assay (Thermo Scientific). After quantification, proteins were denatured for 10 min at 95°C in Laemmli buffer. A total of 15-20 μg of proteins from each condition were separated on SDS-PAGE using 10% acrylamide gels and transferred on 0,22 μm PVDF membrane (BioRad). The membranes were blocked in 5% Non Fat Dry Milk (NFMD) in Tris buffered saline containing 0.1% Tween (TBS-T) for 1h and incubated overnight at 4°C with the following primary antibodies: anti-MDM2 (86934S, Cell Signaling, 1:1000), anti-MDMX (3807946, Millipore,

1:1000), anti-FLT4 (3200622, Millipore, 1:1000), anti-phospho-Tyrosine- (9416S, Cell Signaling 1:1000), anti-p53 (Sc-126, Santa Cruz, 1:1000), and anti-GAPDH (9485, Abcam, 1:1000). After primary antibodies incubation, membranes were washed during 30 min in TBS-T, and incubated with HRP- conjugated secondary antibodies: anti-mouse (Sc-516102, Santa Cruz, 1:2000) or anti-Rabbit (Sc-2357, Santa Cruz, 1:2000) during 1h at room temperature. The membranes were washed for 30 min and bands were detected with the Clarity Max Western ECL Substrate from Bio-Rad Laboratories (Hercules, USA). Results were analyzed by computer-assisted densitometry using ImageQuant LAS-4000 system from GE Healthcare Life Sciences (Mississauga, CAN) and FUJIFILM MultiGauge V3.0.

MDM2 immunoprecipitation and Mass Spectrometry analysis

Following 24h of HEK293T transfection, cells were lysed in IP buffer (Tris 1M, pH=8, NaCl 5M, EDTA 0.5M, Nonidet P-40, 0.5%) containing a cocktail of proteases and phosphatase inhibitors (Roche) at 4°C. The cell lysate was sonicated and centrifuged at 13.000 x g at 4°C, and proteins were quantified using Pierce BCA protein assay (Thermo Scientific). After quantification, 1 mg of protein was incubated with 50 µL of Myc antibody- conjugated beads (Sigma- Aldrich) overnight at 4°C. After centrifugation, the supernatant was removed, and the beads were washed three times with IP buffer. The beads were then denatured for 10 min at 95°C. The IP products were analyzed by Western Blot. MS-MS analysis was performed at Université de Montréal Mass Spectrometry platform (IRIC, Montréal, QC).

Flow cytometry analysis for apoptosis

As previously described, REH cells were seeded until 80% confluency and treated with a gradient of genotoxic drugs (Doxorubicin or Etoposide). After 24h (Etoposide) or 48h (Doxorubicin), cells were washed three times with PBS and incubated at room temperature in Annexin V binding buffer (#42200, Biolegend,) containing APC-conjugated Annexin V antibody (#640919, Biolegend, 1:1000). After 15 min, Annexin V binding buffer containing

DAPI (#422801, Biolegend, 1:500) was added. Apoptosis was immediately measured by Flow cytometry using BD LSR Fortessa. Data analysis was performed using FlowJo Software.

Dual luciferase assay

U2OS were transfected either with FLT4 alone or in combination with MDM2 and MDMX as indicated, alongside with firefly Luciferase reporter plasmids (pGL3-Empty, pg13-p53 binding sites, pg13-mdm2 or pGL3-p21) that were described previously[37]. After 36h of transfection, cells were analyzed using the Dual-Luciferase Assay (Promega) according to the manufacturer's instructions. Renilla luciferase reporter plasmid under the control of the β-globin[37] was used to normalize the data.

Immunofluorescence analysis for MDM2/MDMX complex

24h post-transfection, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-X-100/phosphate-buffered saline. Coverslips were incubated with primary antibody against MDM2 and MDMX, diluted in 1% BSA/phosphate-buffered saline overnight. After washing, coverslips were incubated in a secondary antibody, donkey anti-rabbit AF-647 conjugated antibody (#711-605-152, Jackson ImmunoResearch Laboratories, 1:200) and donkey anti-mouse Cy-3 conjugated antibody (#715-165-150, Jackson ImmunoResearch Laboratories, 1:200) diluted in 1% BSA/phosphate-buffered saline for 1h. Coverslips were washed, and mounted with DAPI. Image acquisition was performed with Zeiss LSM880 multi-photon microscope and data were analyzed using Zeiss Blue software.

Statistical analysis

The results are expressed as mean ± standard error. Statistical significance for multiple comparisons was calculated using ANOVA, while for two-group comparisons was calculated by a Student t-test. P-value < 0.05 was considered statistically significant. Statistical analyses and graphical representation were performed using GraphPad Prism v6.07.

RESULTS

Leukemia patients experience higher levels of FLT4 and VEGF-C expression

We first analyzed the relationship between the expression of FLT4 and VEGF-C and the prognosis of leukemia patients. Using the GEPIA website tool[38], we identified that the mRNA expression levels of FLT4 and VEGF-C were significantly upregulated in Diffuse Large B-cell Lymphoma (DLBC) patients as compared to normal datasets from the cancer genome atlas (TCGA) and the genotype tissue expression (GTEx) project (Fig. 1A). UALCAN database[39] analysis revealed that the FLT4 upregulation is significantly higher in stages 2 and 4 of DLBC as compared to stage 1 (Fig. 1B).

FLT4 overexpression negatively regulates p53 by promoting MDM2/MDMX complex

Hematological malignancies have a relatively low frequency of TP53 mutations. We compared the frequency of p53 mutations amongst various leukemia public cohorts[40–47] in the cBio Cancer Genomics Portal (http://cbioportal.org) (Fig. 2A). The incidence of TP53 mutations in DLBC and ALL (adult or pediatric) was around 20% in DLBC, and less than 10% in ALL in the total number of patients. Given the high wild type status of p53 in leukemia patients (Fig. 2A), we questioned if FLT4 overexpression may promote tumorigenicity by suppressing p53 activity. To gain this general mechanistic insight, we used a cell line harboring wild type p53, U2OS. We found a reduced p53 transcriptional activity in response to FLT4 overexpression by using a p53-responsive luciferase reporter vector (Fig. 2B). To further validate the impact of FLT4 overexpression on reduced p53 activity, U2OS cells were transfected with luciferase reporters for p21 and MDM2, both of which have known p53-binding sites.[48] FLT4 overexpression had significantly decreased the transcriptional activity of p21 and MDM2 promoters (Fig. 2B). These results confirm the suppression of p53 transcriptional activity and two p53 target genes, p21 and MDM2, by FLT4 overexpression.[14]

Our observed p53 suppression suggests that the mechanism of FLT4 may involve the transcription factor's key negative regulatory complex, MDMX and MDM2. We tested this hypothesis by transfecting various combinations of MDMX, MDM2 and FLT4 vector constructs and a p53 reporter vector in U2OS cells, followed by activating p53 activity through 375 μM 5'FU treatment. As expected, we show the suppression of p53 reporter activity by separate MDMX and MDM2 transfections, which had less of an impact individually than their co-expression (Fig. 2C). Interestingly, FLT4 overexpression was able to influence further the combined MDMX and MDM2 suppression of p53 activity.

MDMX and MDM2 are well known to stabilize each other from degradation when they form a heterodimer complex, which consequently accumulates to bind and suppress available p53. After identifying that FLT4 is able to co-modulate p53 activity through the MDM2/MDMX complex, we next studied the influence FLT4 had on stabilizing the complex. To assess this possibility, we transfected increasing amounts of FLT4 vector in HEK293T cells and measured endogenous MDM2 and MDMX expression. Interestingly, FLT4 overexpression increased endogenous MDM2 and MDMX proteins in a concentration-dependent manner, which correlated with an increasing amount of tyrosine phosphorylation (Figure 2D). Similar results were obtained in U2OS cells (Supp. Fig. 1). To further validate this, we transfected different combinations of vectors encoding MDM2, MDMX or FLT4 in HEK293T cells. Our data show that MDMX and MDM2 co-transfection increases MDM2 protein levels compared to an individual MDM2 transfection (Figure 2E). Moreover, the co-transfection of MDMX and MDM2 with FLT4 further induced the amount of MDM2.

FLT4 increase in MDM2 levels led us to evaluate if this effect would decrease p53 protein levels and correlate with the lower levels of p53 activity. While co-transfection of MDM2 and MDMX was able to decrease p53 protein levels compared to their individual transfections, the addition of FLT4 to their co-transfection resulted in a further decrease in p53

levels (Fig. 2F). Therefore, the difference of MDM2 levels being inversely proportional to the amount of p53 was highest when FLT4 was co-transfected. To investigate whether the decrease in p53 was caused by increased ubiquitination from elevated MDM2, we tested the levels of p53 after transfection with an MDM2 mutant vector that is unable to ubiquitinate (MDM2 C464A) using U2OS cells.[49] Although FLT4 was able to stabilize MDM2 C464A with MDMX, FLT4 had lost the ability to decrease the protein levels of p53 when compared to its co-transfection with wild type MDM2 and MDMX (Fig. 2G), implying the importance of the MDM2 ubiquitin ligase activity in FLT4-mediated decrease in p53.

Taken together, these results suggest that FLT4 overexpression strongly promotes the stabilization of the MDM2/MDMX complex, which subsequently leads to more MDM2 readily available to target p53 for degradation by ubiquitination and decreases its transcriptional activity.

FLT4 overexpression relocalizes the nuclear MDM2/MDMX complex to the cytoplasm

To study the effect that FLT4 has on the localization of MDMX and MDM2, we co-transfected different combinations of MDM2, MDMX and FLT4 encoding vectors in U2OS cells and determined the localization of MDM2 and MDMX by immunofluorescence. As previously shown, MDMX alone was found in the cytoplasm, whereas MDM2 alone was localized in the nucleus.[50] When MDMX and MDM2 were co-expressed, MDMX relocalized in the nucleus with MDM2 (Fig. 3). However, when FLT4 was overexpressed, we detected the MDM2/MDMX complex in the cytoplasm, suggesting that FLT4 was responsible for a localization shift of the heterocomplex. Our immunofluorescence results also confirmed the protein stability from our Western Blot studies, where MDM2 is stabilized by MDMX and furthermore by FLT4. Taken together, these results show that FLT4 overexpression promotes the stability of the MDM2/MDMX complex and relocalizes it in the cytoplasm.

FLT4 induces phosphorylation of MDMX on Ser-314 to affect MDM2/MDMX heterodimerization

Having shown that FLT4 overexpression induces an increase in the MDM2/MDMX complex, we aimed to investigate their molecular events. To substantiate the impact of FLT4 on MDMX and MDM2, HEK293T cells were transfected with Myc-MDM2 and MDMX with or without FLT4 and then subjected to immunoprecipitation (IP) using Myc antibody-conjugated beads. As shown in Fig. 4A, the presence of FLT4 caused a greater pulldown of MDMX along with MDM2, suggesting a higher level of heterodimerization and subsequent stability of MDM2 (Fig. 4A). A Mass Spectrometry analysis (MS/MS) was performed on the IP products to identify phosphosites on either MDMX or MDM2, which may influence their heterodimerization. Interestingly, our data show that FLT4 overexpression induced phosphorylation of a serine residue on MDMX at position 314, Ser-314, which we have previously identified as a site involved in the MDM2/MDMX stability by two other RTKs, Her4 and Axl.[36,51] To validate if MDMX Ser-314 had the same impact on FLT4 signaling as our previous RTKs, we transfected HEK293T cells with a combination of FLT4 and MDM2 vectors, and either a wild type MDMX or an MDMX mutant vector that contains a 314 Serine residue replaced with Alanine to impede its phosphorylation (MDMX S314A). Regardless of the combination of MDMX S314A, MDM2 and FLT4 co-expression, we observed a decrease in MDMX S314A stability compared to those transfections with wild type MDMX (Fig. 4B and Supp. Fig. 2), which consequently affected MDM2 levels. Furthermore, when Myc-MDM2 was immunoprecipitated using Myc conjugated beads, we revealed that the Ser-314 phosphosite affected the affinity between MDMX and MDM2, both with or without FLT4 co-transfections (Fig. 4C). This suggests that FLT4 induces phosphorylation of Ser-314 on MDMX, resulting in an increase in MDMX protein level that would be available to bind with MDM2. (Fig. 4C).

To investigate which kinase was responsible for the novel phosphorylation, we previously analyzed the MDMX Ser-314 site using the kinase prediction software GPS to identify potential candidates that mediate the FLT4 signal. The search criteria used were highly stringent, and the software identified CDK4/6 as a candidate kinase. To verify this prediction, HEK293T cells were pretreated with Palbociclib, a specific inhibitor for CDK4/6, which impeded the ability of FLT4 to stabilize the MDMX-MDM2 complex, as shown in Fig. 4D.

Therefore, our findings suggest that FLT4's oncogenic behavior is partly due to its ability to phosphorylate MDMX at Ser-314 in a CDK4/6-dependent manner. This results in increased stability of both MDMX and the MDMX-MDM2 heterocomplex, ultimately leading to p53 inactivation.

Activation of FLT4 in leukemic cells stabilizes the MDM2/MDMX complex leading to p53 decrease

To add relevance to our overexpression experiments, we examined the impact of stimulating endogenous FLT4 on the signaling mechanisms found in leukemia. Specifically, we used the ALL cell line REH due to their wild type p53 and high FLT4 expression compared to other known ALL cell lines, which express a p53 mutant (Fig. 5A). To activate endogenous FLT4, we treated REH leukemic cells with 100 ng/mL of its ligand, VEGF-C, at different time points. FLT4 activity was measured through total tyrosine phosphorylation and evaluated for its potential effect on the stability of intracellular MDM2/MDMX and p53 levels. Over a span of about 3 hours, VEGF-C was able to stimulate total tyrosine phosphorylation as early as one hour, which corresponded to a gradual increase in MDMX and MDM2 and a decrease in p53 levels (Fig. 5B and Supp. Fig. 3A). Interestingly, our 5min intervals of VEGF-C treatment revealed a cyclic increase in MDM2/MDMX followed by a decrease in p53, and vice versa. To confirm the effect of FLT4 on MDM2/MDMX complex, we treated the cells with a specific inhibitor of FLT4, MAZ51. A 30-minute pre-treatment with MAZ51 reversed the FLT4 effect

by decreasing MDM2 and MDMX as early as 30 min and restoring p53 levels (Fig. 5C and Supp. Fig. 3B).

To validate our results, we stably transduced FLT4 in REH cells. We similarly found FLT4 overexpression to increase the protein levels of MDMX and MDM2 while decreasing that of p53 (Fig. 5D). The CDK4/6 pathway was also implicated in the FLT4 stability of MDMX and MDM2 since Palbociclib reversed the effect (Fig. 5E).

Taken together, the influence of the FLT4-CDK4/6 pathway on MDM2/MDMX/p53 axis is conserved within an *in vitro* leukemic model.

The effect of FLT4 on MDM2/MDMX and p53 enhances cell survival and increased resistance to chemotherapy

After showing that FLT4 has the ability to stabilize the MDM2/MDMX complex, we aimed to investigate whether this impact of FLT4 on leukemic cells would have any implications on p53 cell death and response to DNA-damaging agents. First, we observed that the FLT4-induced REH cells led to an increase in cell proliferation (Fig. 6A) and colony formation (Fig. 6B). To assess the effect of FLT4 in response to DNA damage, we treated the REH overexpressing FLT4 cells with genotoxic drugs commonly used in the treatment of leukemia, Doxorubicin and Etoposide, for 5h and 3h respectively (Fig. 6C and 6F). The results showed that MDM2 was higher in cells overexpressing FLT4 as compared to the control. Furthermore, cells overexpressing FLT4 were shown to have lower levels of p53 in response to DNA damage in both genotoxic treatments (Fig. 6C and 6F). Since p53 regulates cell death, we studied the effects of a p53 decrease on apoptosis activation in response to DNA damage stress. After treatment with genotoxic drugs, we performed staining with Annexin V and DAPI and measured the amount of apoptosis. Interestingly, cells overexpressing FLT4 showed lower levels of apoptosis compared to the controls with both Doxorubicin and Etoposide (Fig 6D and 6G), which was concentration-dependent (Fig. 6E and H).

Collectively, these results show that FLT4 activation increases the proliferation of leukemic cells. Furthermore, the constitutive activation of FLT4 decreases the sensitivity of leukemic cells to the DNA-damage induced- apoptosis, therefore increasing their survival.

Targeting CDK4/6 acts as a Therapeutic Strategy to Overcome FLT4-Induced Chemoresistance in Leukemia

As our main finding, we observed that FLT4 phosphorylation of a novel Ser-314 site on MDMX, mediated by CDK4/6, is partly responsible for the increased amount of the MDMX-MDM2 complex and subsequent decrease in p53. As a therapeutic option, we tested if blocking CDK4/6 could decrease the survival of FLT4 overexpressing leukemic cells and succumb them to DNA damaging therapy. First, we observed that the increased proliferation induced by FLT4 overexpression on REH cells was significantly reduced by the pretreatment with the CDK4/6 inhibitor, Palbociclib (Fig. 7A). Furthermore, Palbociclib was able to succumb FLT4-REH cells to a higher percentage of apoptosis in response to Doxorubicin (Fig. 7B).

In summary, our findings established that FLT4 exhibits its oncogenic properties by phosphorylating MDMX on Ser-314 in a manner dependent on CDK4/6. In turn, this leads to elevated stability of MDM2, which results in an increase in the MDMX-MDM2 heterocomplex, ultimately resulting in lower levels of p53 protein and activity, as illustrated in Figure 8.

DISCUSSION

Chemotherapy depends on the activation of p53, a master regulator of cellular stress and DNA damage.[11] Disruption of p53 plays a role in the cell during cancer development and its response to DNA-damaging therapies. While p53 mutations are frequent in solid tumors, p53 mutations occur in less than 10% of leukemia.[19] Thus, inactivation rather than mutations could play a role in the proliferation of leukemic cells and chemoresistance. Therefore, it is crucial to

understand the mechanisms of chemoresistance in leukemia to develop better and more effective treatments.

RTK deregulation is common in several cancers, allowing tumor cells to increase their proliferation and survival.[29,52] An understudied phenomena is the overexpression of the RTK FLT4 and its specific ligand VEGF-C in leukemia, as well as its association with treatment failure.[30] Interestingly, this association had been predominantly shown in pediatric acute lymphoid leukemia.[3] However, to date, the role of FLT4 in regulating p53 has not been studied. This suggests that the pro-survival pathway of FLT4 may be impeding the ability of p53 to suppress its tumor activity but also to respond to DNA damaging therapies. In this study, we provide evidence that the activation of FLT4 in leukemic cells, either through overexpression or VEGF-C stimulation, stabilizes and relocates MDM2 and MDMX into the cytoplasm and reduces the levels of p53. Additionally, we found that the ubiquitination activity of MDM2 plays a crucial role in reducing p53 levels via FLT4. Since MDM2 and MDMX proteins are the main negative regulators of p53, these findings suggest that the cytoplasmic relocalization of MDM2/MDMX may be promoting the nuclear export of p53 for its ubiquitination-targeted degradation. Consequently, the decrease of p53 in leukemic cells correlated with an increase in the survival of leukemic cells under normal and genotoxic conditions, which resulted in a greater colony size. Hence, we provide a mechanism through FLT4 that contributes to tumorigenesis and response to DNA damage by modulating the MDM2/MDMX/p53 axis.

Under VEGF-C stimulation over short periods of time, oscillations in the stability of MDM2 and MDMX and p53 levels are observed possibly due to the dynamic interaction between p53 and MDM2. The tumor suppressor protein p53 is a master transcriptional regulator of the response of human cells to DNA damage. Upon cellular exposure to DNA damage, p53 stabilization leads to the transcriptional induction of hundreds of genes involved in DNA repair, cell-cycle arrest, apoptosis, and cellular senescence.[11] In addition, p53 regulates the expression

of proteins involved in controlling its levels, including MDM2 and MDMX, which tags p53 for proteasomal-dependent degradation and consequently lower MDM2/MDMX expression.[14] This oscillatory dynamics of p53 inhibition generated by the interaction of p53 and MDM2 creates a negative feedback loop. Therefore, VEGF-C stimulation of MDM2/MDMX stability will lead to p53 degradation, reducing the transcription of MDM2 and MDMX, which in turn positively upregulate p53 levels. The interaction of p53 and MDM2 generates oscillatory dynamics of p53 activation characterized by a stereotyped frequency and noisy amplitude. Fluctuations in the oscillatory pattern of p53 trigger a sharp switch between its activation and inhibition, leading to escape from cell-cycle arrest. Transient perturbation of p53 stability mimics the noise in individual cells and is sufficient to trigger escape from arrest.[53] Thus, self-reinforcing circuitry that mediates cell-cycle transitions can translate small fluctuations in p53 signaling into large phenotypic changes of oncogenic propagation. In short, oscillations in the stability of MDM2 and MDMX and p53 levels are observed under VEGF-C stimulation over short periods due to the dynamic interaction between p53 and MDM2.

In order to dissect the molecular mechanisms of p53 regulation by FLT4, we performed a MS/MS analysis on the MDM2/MDMX complex. Interestingly, the phosphosite Ser-314 on MDMX was increased in the presence of FLT4. Ser-314 is located on the zinc finger domain of MDMX, a region that does not contact MDM2 and is known to play a role in kinase signaling.[13] This implies that the FLT4-mediated signal directly affects MDMX to indirectly affect MDM2, possibly regulating MDMX stability through phosphorylation. The same phosphosite was shown by our previous studies to be implicated in the stabilization of MDMX by the RTKs HER4 and AXL in the context of breast cancer and melanoma, respectively.[36,51] The importance of Ser-314 for the stability of MDMX in our new model of FLT4 was confirmed using a mutant MDMX, where the Serine 314 site was replaced by Alanine, which led to a reduction in MDMX and a loss in the FLT4-induced stabilization of the heterodimer complex.

One of the kinases predicted to be responsible for the phosphorylation of MDMX Ser-314 was CDK4/6, which is a master regulator of the cell cycle that regulates the critical checkpoint in the G1-S transition needed for the progression of the cell cycle.[54] It is noteworthy that certain cancers, including hematological malignancies, possess a functional CDK4/6 pathway alongside elevated levels of MDM proteins and retain a wild type p53 and that CDK4/6 inhibitors can impede the cell cycle progression of cancerous cells. In fact, we observed that CDK4/6 inhibition reduced MDMX levels and increased susceptibility to apoptosis in FLT4-overexpressing cells.

While targeting RTKs with inhibitors has shown limited success as a strategy in cancer treatment, their effects on the p53 regulatory unit have not been fully explored therapeutically. Our findings suggest that the stabilization of the MDMX-MDM2 complex is the converging point of oncogenic signals, mediated by the activation of FLT4 and CDK4/6 kinase and leading to enhanced suppression of p53 activity. Our proposed model takes into account our results and the function of the MDMX zinc finger domain in transmitting kinase signaling. In our proposed model, the activation of CDK4/6 by FLT4 triggers the phosphorylation of MDMX at Ser-314, which causes it to undergo a conformational change that enhances its stability. Subsequently, MDMX binds to and stabilizes MDM2, which leads to the stabilization of the MDM2/MDMX complex and, as a consequence, inhibits the tumor suppression and DNA-damaging response of p53 by shuttling it to the cytoplasm and ubiquitinating it for degradation (Fig. 8).

In conclusion, ALL harbor a wild type p53 and often have elevated levels of FLT4/VEGF-C, correlating with poor prognosis. In addition, VEGF-C treated leukemic cells induce mitogenic effects and protect against apoptosis. It is possible ALL share a common mechanism of p53 suppression through the FLT4/VEGF-C axis to support their carcinogenic qualities and therapy resistance. For these reasons, our research proposes an innovative way to

reactivate p53 in pediatric leukemia through the pharmacological inhibition of FLT4 signaling as a novel treatment of pediatric leukemia.

AUTHOR CONTRIBUTION STATEMENT

C.G. conceived and designed the experiments; M.D., D.H., C.M., and C.G. performed the experiments; M.D., D.H., J.B.C., and C.G. analyzed the results and wrote the manuscript; J.B.C., E.B.A., B.L., X.L., and C.G. discussed and analyzed the experiments; All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

All data generated or analyzed during this study are present in this published article. Any additional information pertinent to the data may be requested from the corresponding author.

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FIGURE LEGENDS

Figure 1: FLT4 and its ligand VEGF-C are upregulated in patients and are associated with cancer progression. A) FLT4 and VEGF-C mRNA expression in healthy and Diffuse Large B-cell Lymphoma (DLBC) patients. The data were generated using Gene Expression Profiling Interactive Analysis (GEPIA). DLBC (n=47), Healthy (n=337), *p<0.01 (Log_2FC 0.6). **B)** FLT4 expression in DLBC patients at individual cancer stages. The data were generated using the ULCAN-TCGA database. Stage 1 (n=8), stage 2 (n=17), stage 3 (n=4), Stage 4 (n=12). *p<0.05 vs. stage 1.

Figure 2: FLT4 overexpression negatively regulates p53 expression and subsequently downregulates its target genes. A) The frequency of p53 mutation in Diffuse Large B Cell lymphoma (DLBC) patients *(DFCI, Nat Med, 2018*), *(Broad, PNAS, 2012), (BCGSC, Blood, 2013*), *(TCGA, PanCancer Atlas), (Duke, Cell, 2017),* ALL patients *(St Jude, Nat Genet, 2016), (St Jude, Nat Genet, 2015),* and pediatric ALL patients (TARGET, 2018) – across public datasets in https://www.cbioportal.org/. **B)** U2OS cells were transfected with an empty or FLT4 expression plasmid along with a pg13 luciferase reporter vector or a luciferase reporter for p21 or MDM2 to determine p53 target genes activity. **C)** U2OS cells were transfected with different combinations of FLT4, MDM2 and MDMX plasmids along with pg13 luciferase reporter vector to determine endogenous p53 under genotoxic conditions (5'FU = 375 μM). **D)** HEK293T cells were transfected with various amounts of FLT4 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. **E)** HEK293T cells were transfected with a combination of FLT4, MDMX and MDM2 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. **F)** U2OS cells were transfected with a combination of FLT4, MDMX or MDM2 expression plasmids for 24h. The cell lysates were blotted for the

indicated antibodies. **G)** U2OS cells were transfected with a combination of FLT4, MDMX, wild type MDM2 or mutant MDM2 C464A expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. *p<0.05, ****p<0.0001.

Figure 3: FLT4 overexpression increases the stability of MDM2/MDMX complex and relocalizes it in the cytoplasm. U2OS cells were transfected with a combination of FLT4, MDMX and MDM2 expression plasmids and immunostained for MDMX (Green) and MDM2 (Red), while the nuclei were stained with DAPI. Scale bar: 20 μm.

Figure 4: FLT4 activation leads to MDMX S314 phosphorylation through the activation CDK4/6 pathway. A) HEK293T cells were transfected with a combination of MDMX, Myc-MDM2 with or without FLT4 and harvested 24h later for immunoprecipitation (IP) with Myc antibody-conjugated beads. Whole Cell Extract (WCE) and Myc IP samples were subjected to Western Blot analysis with the indicated antibodies. **B)** HEK293T cells were transfected with various combinations of wild type MDMX, mutant MDMX S314A, MDM2 and FLT4 plasmids. After 24h of transfection, cells were harvested, and subjected to Western Blot analysis using the indicated antibodies. LE, low exposure. HE, high exposure. **C)** HEK293T cells were transfected with either wild type MDMX or mutant MDMX S314A in combination with Myc-MDM2 and FLT4 plasmids. After 24h of transfection, cell lysates were harvested for IP with Myc-antibody-conjugated beads. WCE and Myc IP samples were subjected to Western Blot analysis with the indicated antibodies. **D)** HEK293T cells were transfected with a combination of MDM2, MDMX and FLT4 plasmids for 24h, then treated with various concentrations of Palbociclib for 24h. Cells were harvested and analyzed by Western Blot using the indicated antibodies.

Figure 5: FLT4 stimulation with VEGF-C requires CDK4/6 to stabilize the MDM2/MDMX complex and p53 decrease in leukemic cells. A) FLT4 expression in leukemic cell lines. Published RNA sequencing data of various *in vitro* ALL models were analyzed for FLT4 expression. The abundance in 'Transcript Per Million' (TPM) was reported as the sum of the TPM values of all its protein-coding transcripts. The threshold level to detect the presence of a transcript for a particular gene was set to ≥ 1 TPM. **B)** REH cells were treated with the FLT4 ligand, VEGF-C (100 ng/mL), for 2h or 3h and then harvested for Western Blot analysis using the indicated antibodies. **C)** REH cells were pre-treated with the FLT4 inhibitor, MAZ51 (5 μM), for 30 min, then treated with 100 ng/ml of VEGF-C for 25 or 30 min. Cell lysates were collected for Western Blot analysis using the indicated antibodies. **D)** REH cells were transduced with FLT4 (vFLT4) or empty vector, then harvested and subjected to Western Blot with the indicated antibodies. **E)** REH transduced cells were pre-treated for 24h with the CDK4/6 inhibitor, Palbociclib (2.5 μM), then harvested for Western Blotted using the indicated antibodies.

Figure 6: FLT4 overexpression decreases p53 and promotes cell survival and resistance to chemotherapy. A) REH cells transduced with FLT4 (vFLT4) or empty vector were seeded in a 6-well plate with a complete medium and counted every day for five consecutive days. **B)** REH transduced cells were plated in 1.2% methyl-cellulose. After two weeks of culture, colonies consisting of at least 50 cells were counted using an inverted microscope. **C)** REH transduced cells were treated with Doxorubicin (50 nM, 5h), then harvested and subjected to Western Blotting for the indicated antibodies. **D, E)** REH transduced cells were treated with 50 nM (D) or various amounts (E) of Doxorubicin for 48h. Cells were stained with Annexin V and

DAPI , and apoptosis was measured by flow cytometry. **F)** REH transduced cells were treated with Etoposide (0.25 μM, 3h), then harvested and subjected to Western Blotting for the indicated antibodies. **G, H)** REH transduced cells were treated with 0.25 μM (G) or various amounts (H) of Etoposide for 24h. Cells were stained with Annexin V and DAPI, and apoptosis was measured by flow cytometry. *p<0.05, **p<0.01.

Figure 7: Inhibition of CDK4/6 prevents FLT4- induction of proliferation and -resistance to DNA damaging apoptosis. A) REH cells transduced with FLT4 (vFLT4) or empty vector were seeded in a 6-well plate in complete medium, then treated with the CDK4/6 inhibitor, Palbociclib (1 μM), at day 0 and counted every day for five consecutive days. **B)** REH transduced cells (vFLT4) were pre-treated with Palbociclib (1 μM) for 24h and then treated with 50 nM of Doxorubicin for 48h. Cells were stained with Annexin V and DAPI, and apoptosis was measured by flow cytometry. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 8: Schematic representation of FLT4-mediated MDM2/MDMX complex stabilization and suppression of p53 activity. VEGF-C triggers FLT4 activation, leading to CDK4/6 activation, which phosphorylates MDMX on Ser-314. As a result, MDMX levels increase and bind to MDM2, stabilizing the MDM2/MDMX complex. This complex binds to p53, facilitating its suppression via reduced transcriptional activity or enhanced export to the cytoplasm for proteasome degradation. Consequently, p53 inactivation promotes their survival, proliferation, and resistance to chemotherapy-induced apoptosis. *The figure was created in BioRender.com*

Supplementary Figure 1 : U2OS cells were transfected with various amounts of FLT4 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies.

Supplementary Figure 2: HEK293FT cells were transfected with MDMX WT or MDMX S314A expression plasmids for 24h. The cell lysates were blotted for the indicated antibody.

Supplementary Figure 3: A) At various time points, REH cells were treated with the FLT4 ligand, VEGF-C (100 ng/mL), then harvested and subjected to Western Blot with the indicated antibodies. **B)** REH cells were pre-treated for 30 min with the FLT4 inhibitor, MAZ51 (5 μM), then treated with 100 ng/ml of VEGF-C for 2h. The cells were harvested for Western Blot with the indicated antibodies.

Figures

Figure 1

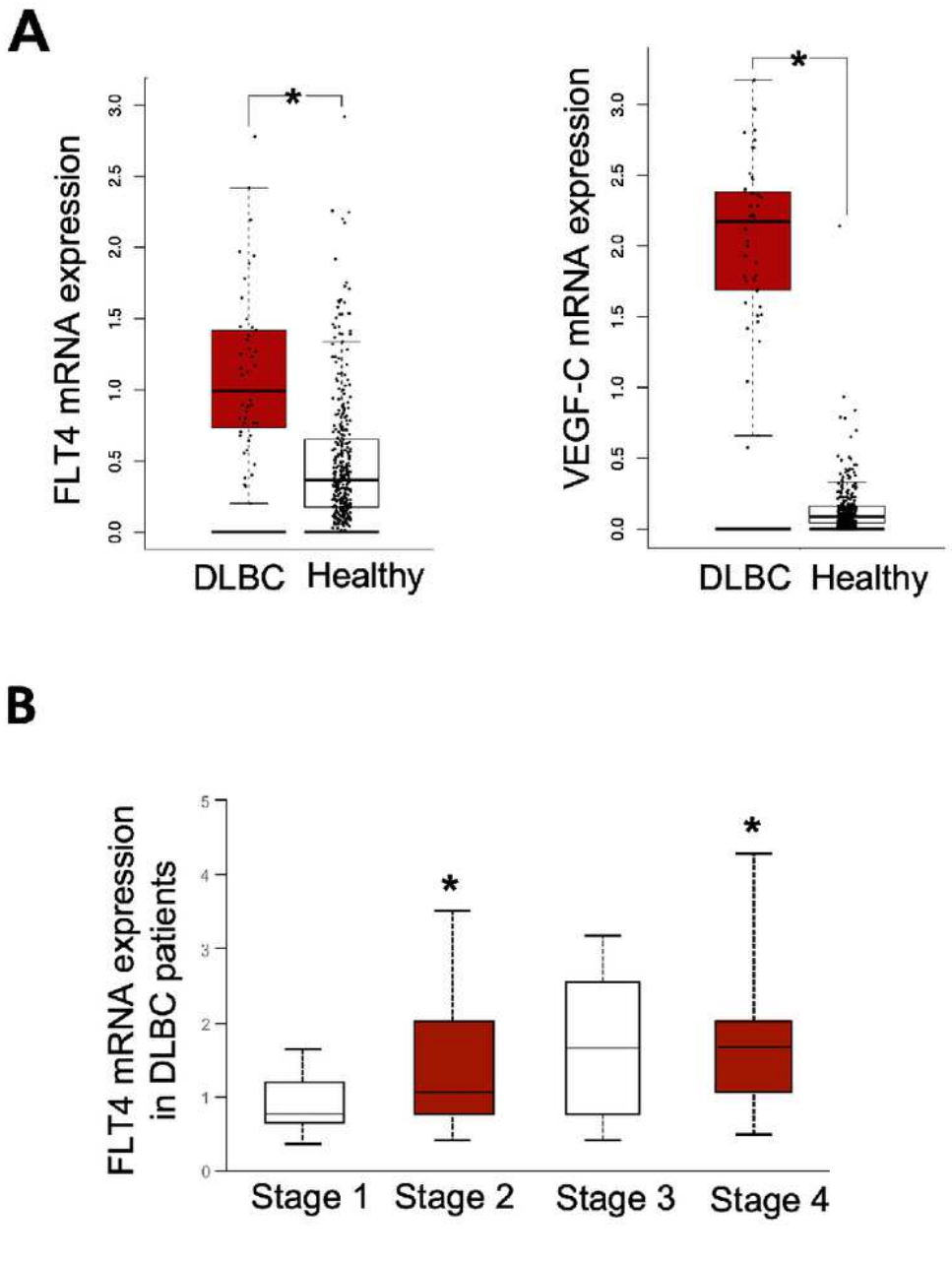

Figure 1

FLT4 and its ligand VEGF-C are upregulated in patients and are associated with cancer progression. A) FLT4 and VEGF-C mRNA expression in healthy and Diffuse

Large B-cell Lymphoma (DLBC) patients. The data were generated using Gene Expression Profiling Interactive Analysis (GEPIA). DLBC (n=47), Healthy (n=337), *p<0.01 (Log2FC 0.6). B) FLT4 expression in DLBC patients at individual cancer stages. The data were generated using the ULCAN-TCGA database. Stage 1 (n=8), stage 2 (n=17), stage 3 (n=4), Stage 4 (n=12). *p<0.05 vs. stage 1.

Figure 2

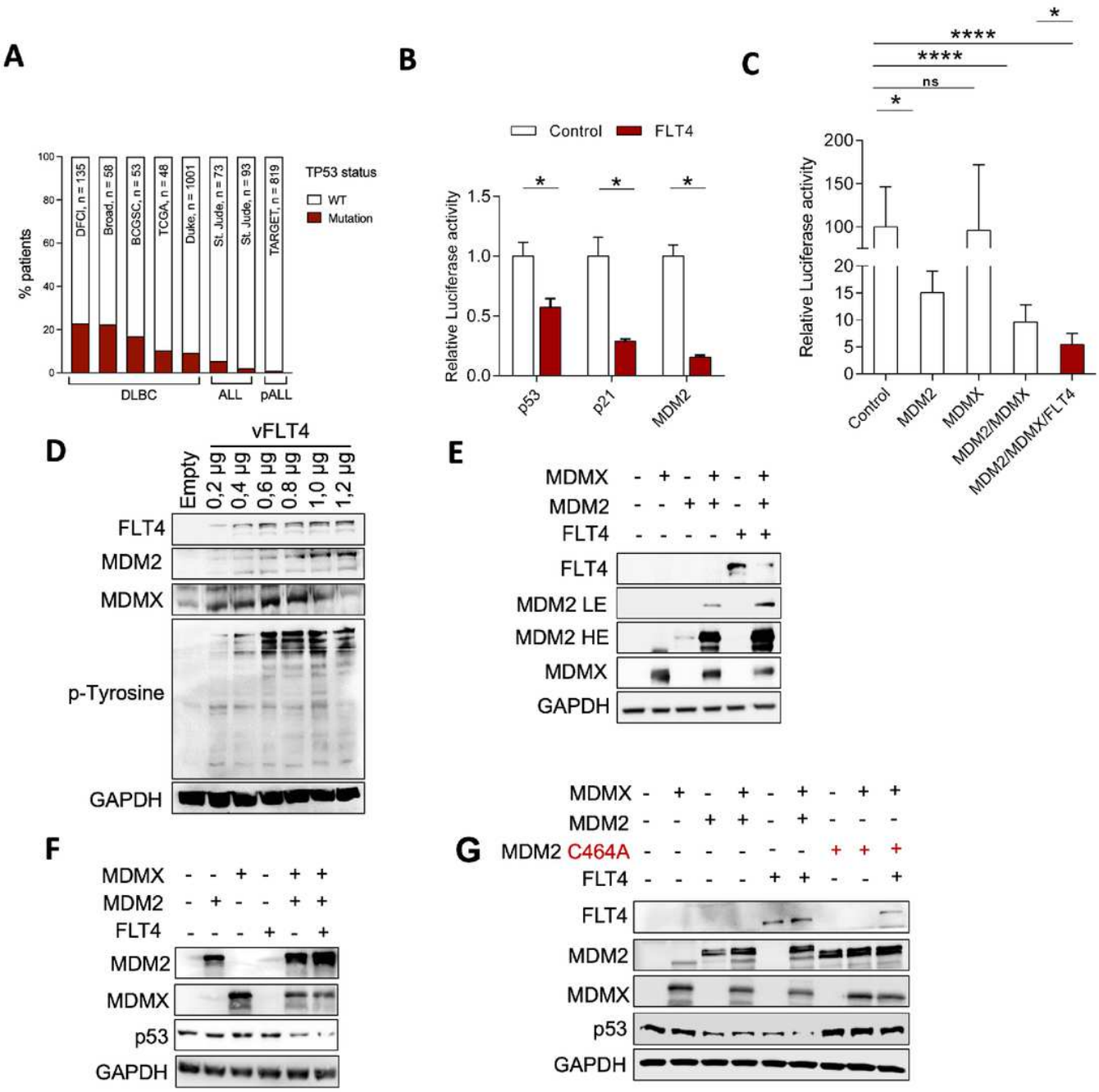

Figure 2

FLT4 overexpression negatively regulates p53 expression and subsequently downregulates its target genes. A) The frequency of p53 mutation in Diffuse Large B Cell lymphoma (DLBC) patients (DFCI, Nat Med, 2018), (Broad, PNAS, 2012), (BCGSC, Blood, 2013), (TCGA, PanCancer Atlas), (Duke, Cell, 2017), ALL patients (St Jude, Nat Genet, 2016), (St Jude, Nat Genet, 2015), and pediatric ALL patients (TARGET, 2018) – across public datasets in https://www.cbioportal.org/. B) U2OS cells were transfected with an empty or FLT4 expression plasmid along with a pg13 luciferase reporter vector or a luciferase reporter for p21 or MDM2 to determine p53 target genes activity. C) U2OS cells were transfected with different combinations of FLT4, MDM2 and MDMX plasmids along with pg13 luciferase reporter vector to determine endogenous p53 under genotoxic conditions (5'FU = 375 μM). D) HEK293T cells were transfected with various amounts of FLT4 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. E) HEK293T cells were transfected with a combination of FLT4, MDMX and MDM2 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. F) U2OS cells were transfected with a combination of FLT4, MDMX or MDM2 expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. G) U2OS cells were transfected with a combination of FLT4, MDMX, wild type MDM2 or mutant MDM2 C464A expression plasmids for 24h. The cell lysates were blotted for the indicated antibodies. *p<0.05, ****p<0.0001.

Figure 3

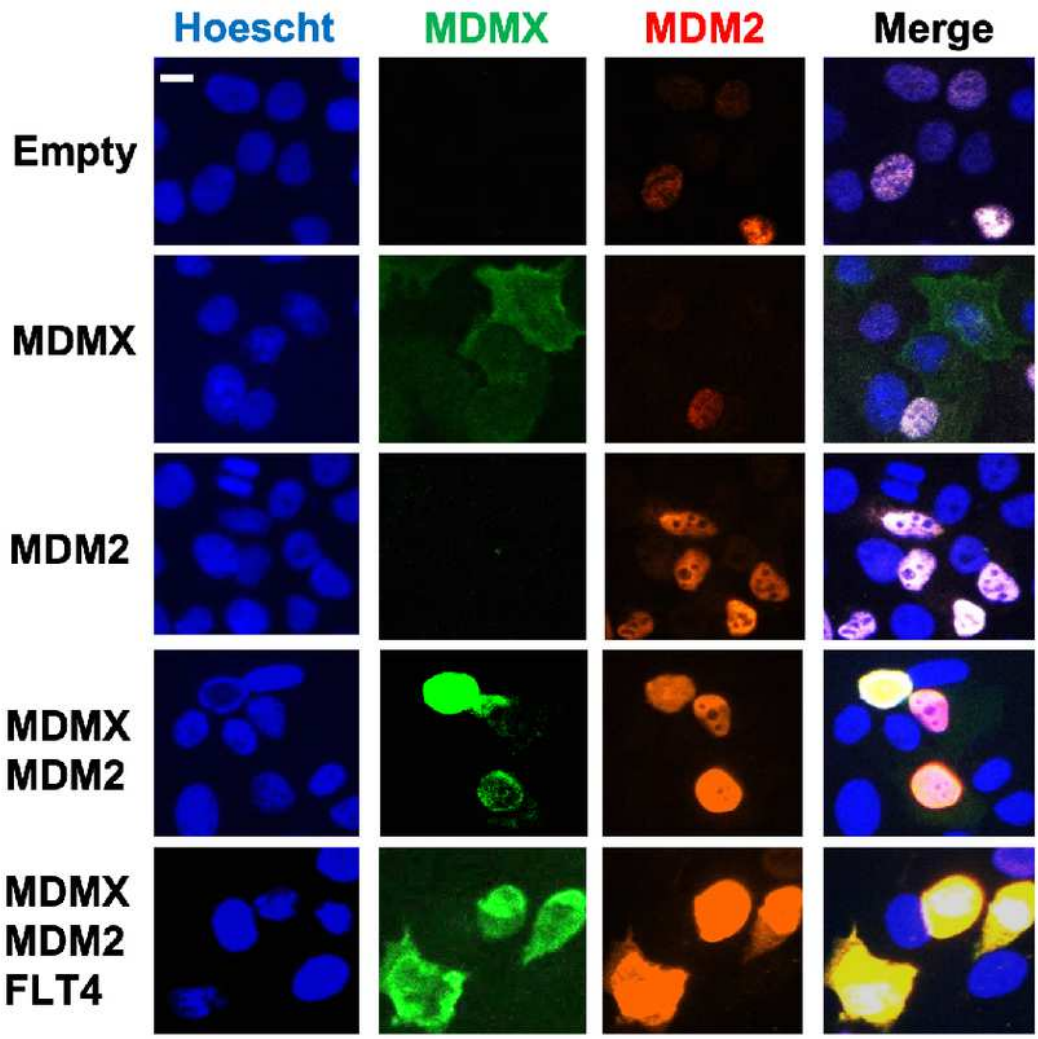

Figure 3

FLT4 overexpression increases the stability of MDM2/MDMX complex and relocalizes it in the cytoplasm. U2OS cells were transfected with a combination of FLT4, MDMX and MDM2 expression plasmids and immunostained for MDMX (Green) and MDM2

(Red), while the nuclei were stained with DAPI. Scale bar: 20 μm.

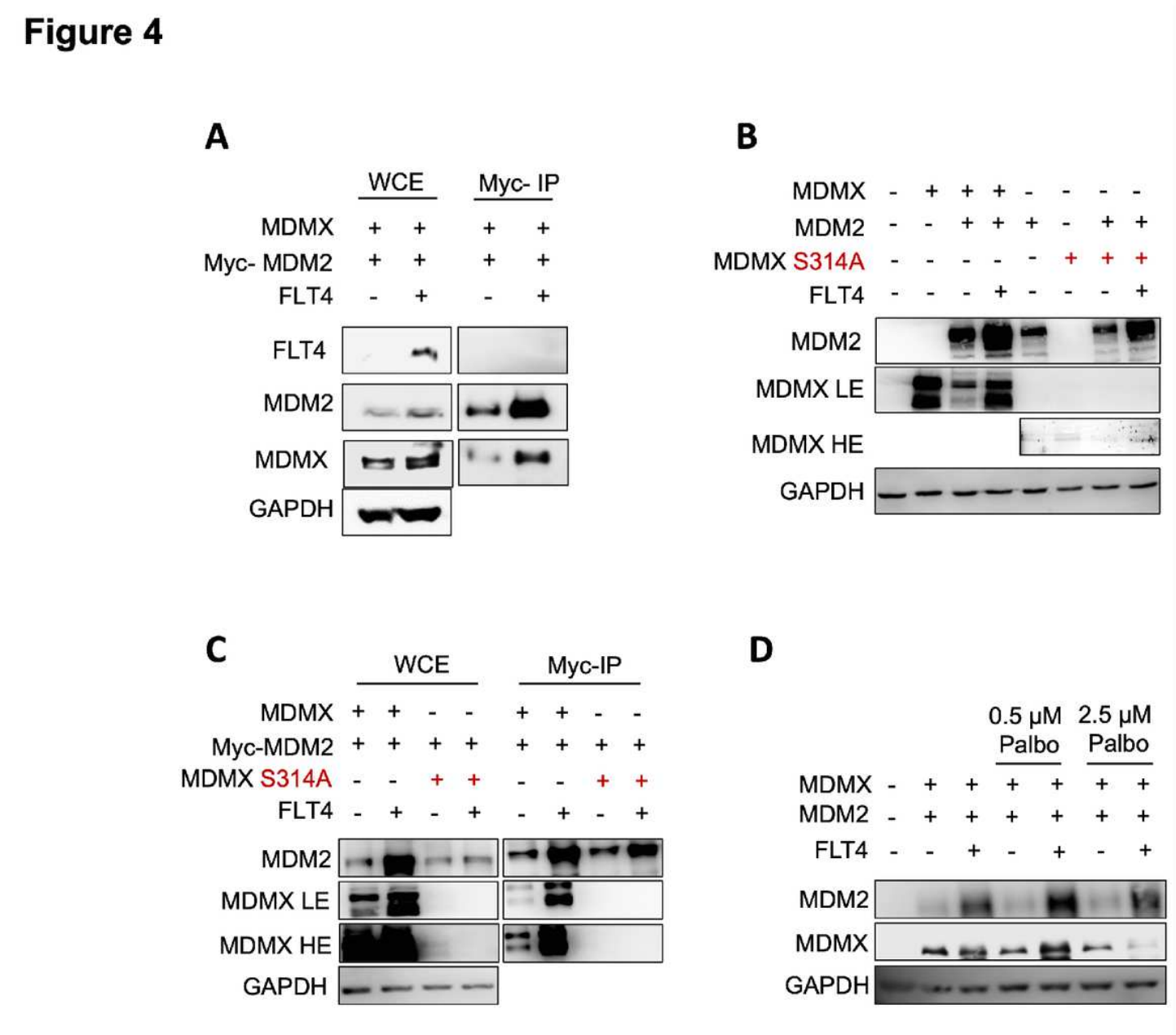

Figure 4

FLT4 activation leads to MDMX S314 phosphorylation through the activation CDK4/6 pathway. A) HEK293T cells were transfected with a combination of MDMX, Myc-MDM2 with or without FLT4 and harvested 24h later for immunoprecipitation (IP) with Myc antibody-conjugated beads. Whole Cell Extract (WCE) and Myc IP samples were subjected to Western Blot analysis with the indicated antibodies. B) HEK293T cells were transfected with various combinations of wild type MDMX, mutant MDMX S314A, MDM2 and FLT4

plasmids. After 24h of transfection, cells were harvested, and subjected to Western Blot analysis using the indicated antibodies. LE, low exposure. HE, high exposure. C) HEK293T cells were transfected with either wild type MDMX or mutant MDMX S314A in combination with Myc-MDM2 and FLT4 plasmids. After 24h of transfection, cell lysates were harvested for IP with Myc-antibody-conjugated beads. WCE and Myc IP samples were subjected to Western Blot analysis with the indicated antibodies. D) HEK293T cells were transfected with a combination of MDM2, MDMX and FLT4 plasmids for 24h, then treated with various concentrations of Palbociclib for 24h. Cells were harvested and analyzed by Western Blot using the indicated antibodies.

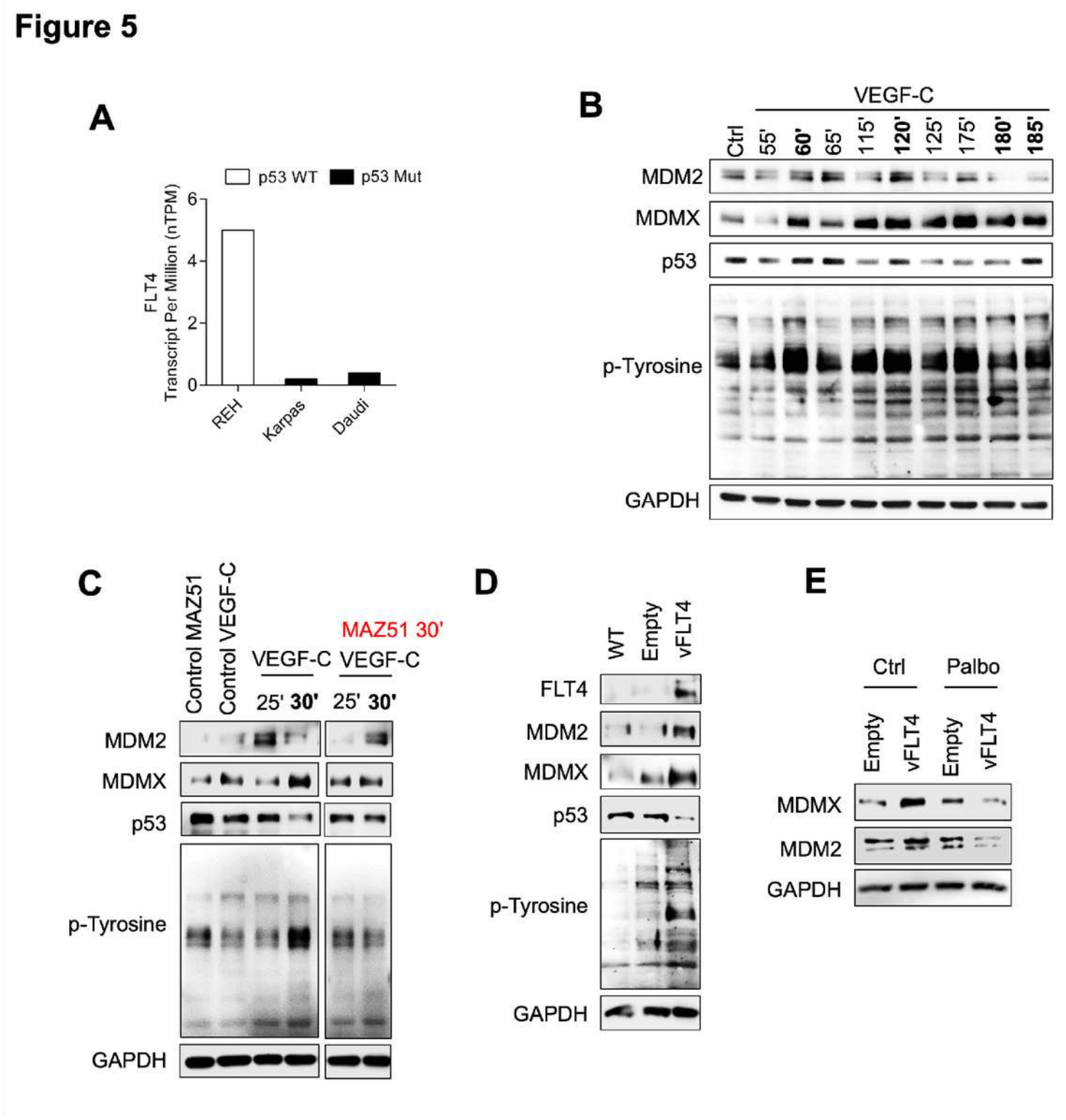

Figure 5

FLT4 stimulation with VEGF-C requires CDK4/6 to stabilize the MDM2/MDMX complex and p53 decrease in leukemic cells. A) FLT4 expression in leukemic cell lines. Published RNA sequencing data of various in vitro ALL models were

analyzed for FLT4 expression. The abundance in 'Transcript Per Million' (TPM) was reported as the sum of the TPM values of all its protein-coding transcripts. The threshold level to detect the presence of a transcript for a particular gene was set to ≥ 1 TPM. B) REH cells were treated with the FLT4 ligand, VEGF-C (100 ng/mL), for 2h or 3h and then harvested for Western Blot analysis using the indicated antibodies. C) REH cells were pre-treated with the FLT4 inhibitor, MAZ51 (5 μM), for 30 min, then treated with 100 ng/ml of VEGF-C for 25 or 30 min. Cell lysates were collected for Western Blot analysis using the indicated antibodies. D) REH cells were transduced with FLT4 (vFLT4) or empty vector, then harvested and subjected to Western Blot with the indicated antibodies. E) REH transduced cells were pre-treated for 24h with the CDK4/6 inhibitor, Palbociclib (2.5 μM), then harvested for Western Blotted using the indicated antibodies.

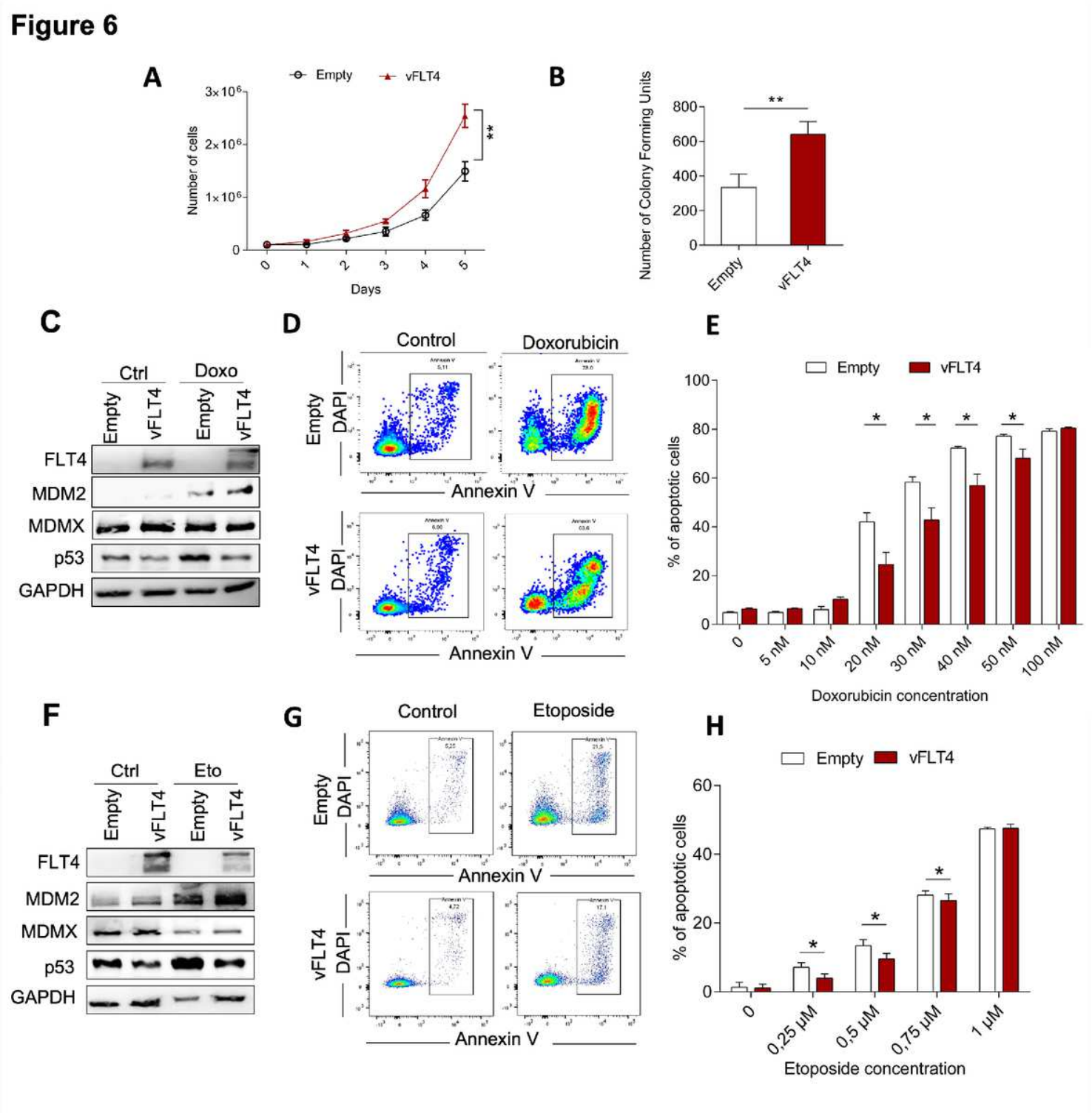

Figure 6

FLT4 overexpression decreases p53 and promotes cell survival and resistance to chemotherapy. A) REH cells transduced with FLT4 (vFLT4) or empty vector were seeded in a 6-well plate with a complete medium and counted every day for five consecutive days. B) REH transduced cells were plated in 1.2% methyl-cellulose. After two weeks of culture,

colonies consisting of at least 50 cells were counted using an inverted microscope. C) REH transduced cells were treated with Doxorubicin (50 nM, 5h), then harvested and subjected to Western Blotting for the indicated antibodies. D, E) REH transduced cells were treated with 50 nM (D) or various amounts (E) of Doxorubicin for 48h. Cells were stained with Annexin V and DAPI , and apoptosis was measured by flow cytometry. F) REH transduced cells were treated with Etoposide (0.25 μM, 3h), then harvested and subjected to Western Blotting for the indicated antibodies. G, H) REH transduced cells were treated with 0.25 μM (G) or various amounts (H) of Etoposide for 24h. Cells were stained with Annexin V and DAPI, and apoptosis was measured by flow cytometry. *p<0.05, **p<0.01.

Figure 7

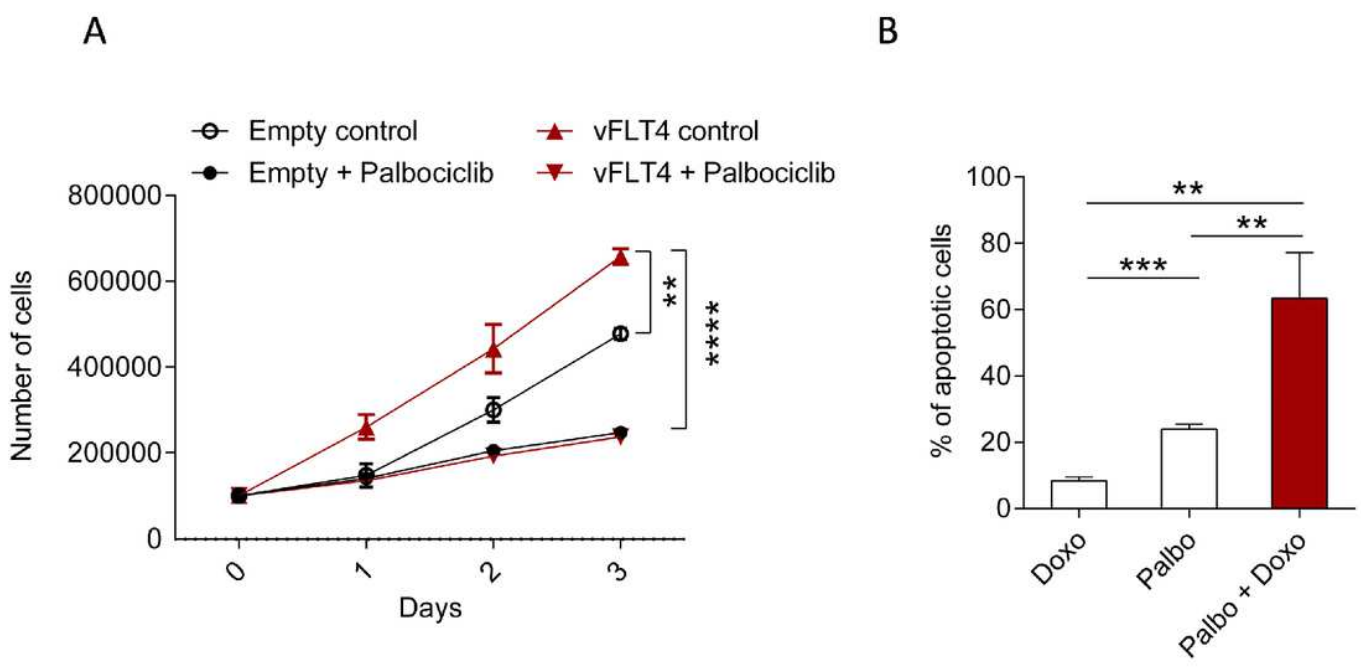

Figure 7

Inhibition of CDK4/6 prevents FLT4- induction of proliferation and -resistance to DNA damaging apoptosis. A) REH cells transduced with FLT4 (vFLT4) or empty vector were seeded in a 6-well plate in complete medium, then treated with the CDK4/6 inhibitor, Palbociclib (1 μM), at day 0 and counted every day for five consecutive days. B) REH

transduced cells (vFLT4) were pre-treated with Palbociclib (1 μM) for 24h and then treated with 50 nM of Doxorubicin for 48h. Cells were stained with Annexin V and DAPI, and apoptosis was measured by flow cytometry. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 8

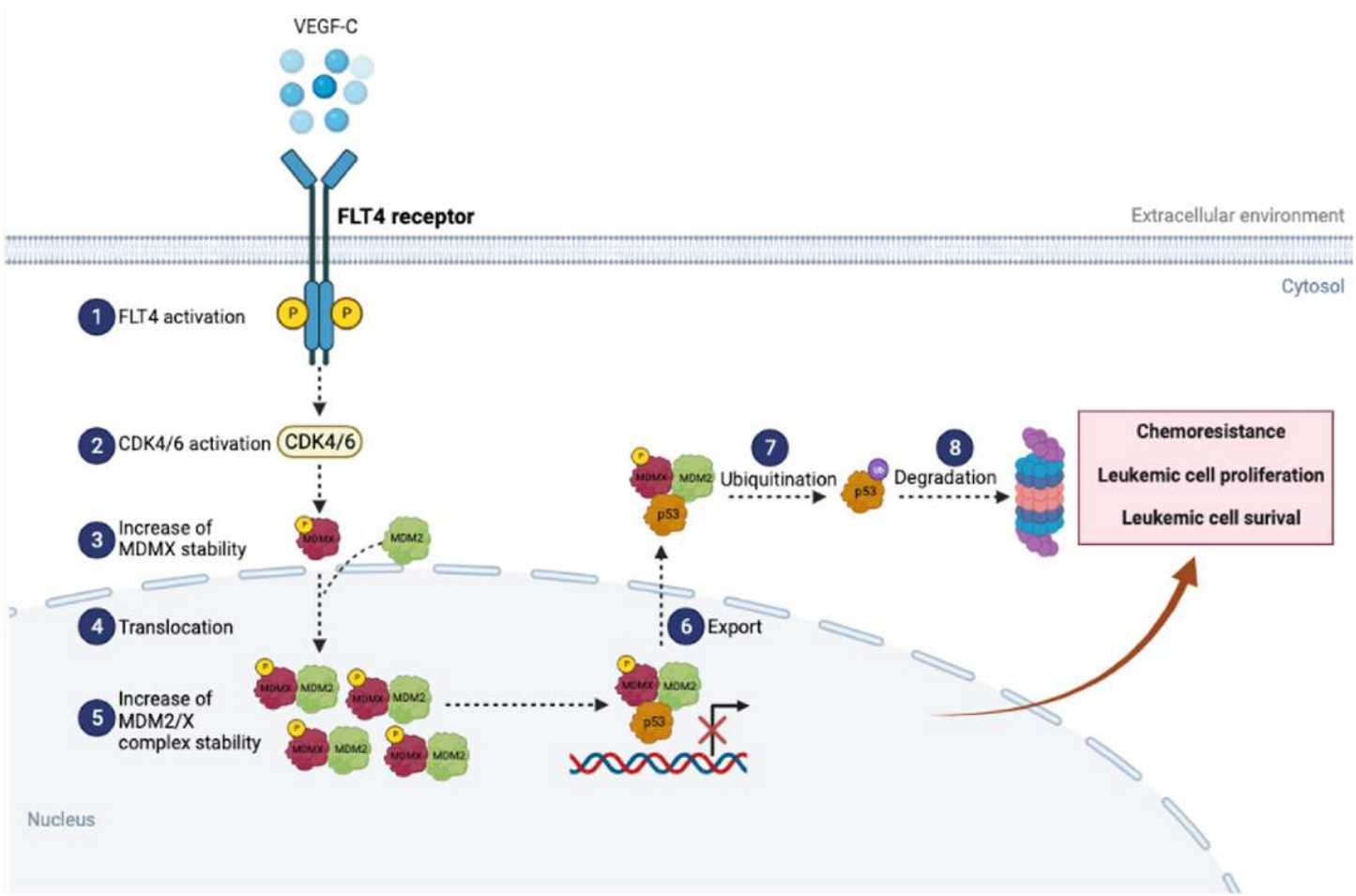

Figure 8

Schematic representation of FLT4-mediated MDM2/MDMX complex stabilization and suppression of p53 activity. VEGF-C triggers FLT4 activation, leading to CDK4/6 activation, which phosphorylates MDMX on Ser-314. As a result, MDMX levels increase and bind to MDM2, stabilizing the MDM2/MDMX complex. This complex binds to p53, facilitating its suppression via reduced transcriptional activity or enhanced export to the cytoplasm for proteasome degradation. Consequently, p53 inactivation promotes their survival,

proliferation, and resistance to chemotherapy-induced apoptosis. The figure was created in BioRender.com

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pdf
- SupplementaryFigure2.pdf
- SupplementaryFigure3.pdf