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Transphosphorylation of FLT3 proteins

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Letter for Haematologica

Ligand binding of receptor tyrosine kinases (RTKs) results in a conformational alteration of the extracellular domain and induces receptor homooligomerization (1). Subsequently, interactions between adjacent cytoplasmic domains are stabilized which lead to their intermolecular phosphorylation. This so-called transphosphorylation activates the kinase domain of the binding partner (2). Transphosphorylation was first shown for epidermal growth factor receptor (EGFR) and insulin receptor (IR). By using a kinase-dead K721A mutant, it has been demonstrated that in the presence of wild-type EGFR and EGF stimulation, the kinase-dead receptor was phosphorylated on tyrosine residues. This provided direct evidence that EGFR transphosphorylation was mediated by intermolecular (trans) phosphorylation within receptor dimers, rather than by intramolecular events (3). For the demonstration of transphosphorylation of IR, molecules of different species as well as kinase-dead mutants were used (4).

Transphosphorylation occurs in a defined temporal order. Early sites tend to regulate kinase activity by stabilizing its active conformation and later sites regulate pathway choice and signal strength (reviewed in (5). This ordered phosphorylation contributes to signal fidelity graded signaling responses. EGF binding to its receptor results in asymmetric oligomerization. One kinase acts as an activator and the other is the receiver (6). Thus, EGFR can be activated with minimal activation-loop phosphorylation compared to other RTKs. Many RTK like PDGFR (7, 8) and FGFR (9) form symmetric dimers, where both kinases are activated similarly (reviewed in (5)).

For class III RTK like KIT or FLT3 the juxtamembrane domain (JMD) acts autoinhibitory at the kinase. Here transphosphorylation of tyrosine residues in this region disrupts inhibitory contacts and consequently stabilizes the active kinase conformation. JMD extensions by internal tandem duplications (ITD) also consequently result in the de-repression of the kinase, thus resulting in a FLT3 ligand (FL) -independent constitutive activity of the receptor. FLT3 ITD mutations are the most frequent mutation driving the development of Acute Myeloid Leukemia (AML). Kiyoi and co-workers showed that FLT3-ITD proteins form homodimers. By using chemical cross linking they elucidated that FLT3 ITD peptides associated with FLT3 wildtype (WT) (10). Their findings indicated that the FLT3 JMD plays an important role in receptor activation, and that the ITD mutation in the JMD induces ligand-independent receptor activation and probably transactivates FLT3 WT. In general, allelic abundance is relevant for the AML disease development. High allelic abundance (allelic ratio ≥ 0.5) is associated with worse overall survival and relapse risk and results in more aggressive disease biology (11, 12). Thus, for the prognosis of FLT3 ITD AML patients not only the allelic abundance of mutant FLT3 ITD but also the overall expression level of FLT3 is relevant. Moreover, Chen and co-

workers demonstrated that co-expression of FLT3 WT attenuated the effect of FLT3 inhibitors on FLT3 ITD (13). By using epitope-labelled FLT3 variants with altered molecular weights, we provide here evidence for the transphosphorylation of different FLT3 proteins. The transphosphorylation of FLT3 ITD to FLT3 WT is of high medical relevance, since this process consequently results in the FL-independent activation of all FLT3 molecules.

To provide evidence for the transphosphorylation of different FLT3 proteins, we co-expressed FLT3 genes fused to specific epitopes with different molecular weights. In addition, HA-epitope tagging allowed selective immune purification of specific molecule populations. FLT3 proteins can be identified as two abundant forms. While the WT receptor exists mainly as a mature, complex glycosylated cell surface-localized molecule, FLT3 ITD can be predominantly identified in an immature, high-mannose form with an about 20 kDa lower molecular weight (14). As demonstrated in Fig. 1, FLT3 genes were tagged with a 3'-terminally localized region encoding a bivalent GPAC tag consisting of a photo-activatable GFP and mCherry (15). These hybrid genes are expressed as a mature 210 kDa form and a high mannose form of 190 kDa (**Fig. 1A**). Alternatively, the interaction partners were synthesized with a C-terminally localized HA-tag resulting in FLT3-HA proteins with a molecular weight of 150 kDa in its mature and of 130 kDa as immature form. For the elucidation of transphosphorylation, the FLT3-HA proteins are carrying a K644A substitution, resulting in kinase inactive mutants (FLT3-KA-HA) (**Fig. 1A**).

In general, HEK293T cells were co-transfected with the FLT3-enoding plasmid constructs described above, or singular controls of individual plasmids. Following a 4-hour starvation period, cells were lysed, and a two-step immunoprecipitation (IP) was performed to isolate FLT3 proteins (reviewed in **Fig. 1B; Suppl. Table 1**). Anti-HA conjugated agarose beads were used to completely precipitate FLT3-KA-HA proteins. Subsequently remaining GPAC-labelled FLT3 were immunoprecipitated with FLT3 S18 antibodies and Protein A/G beads. For unambiguous identification of proteins immunoblots were carried out after blotting proteins separated on 5.4 % PAA gels. Membranes were first probed for FLT3 phosphorylation using Phospho-FLT3 (Tyr_{591}) antibodies. Subsequently total FLT3 were detected using the anti-FLT3 S18 antibodies.

First, interaction and transphosphorylation of FLT3 WT molecules were elucidated (**Fig. 2A**). FLT3-WT-GPAC and FLT3-WT-KA-HA were co-expressed in HEK293T cells and stimulated with FLT3 ligand. While FLT3-WT-KA-HA alone did not show any phosphorylation, FLT3-WT-GPAC phosphorylated the 150/130 kDa FLT3 WT-KA-HA molecules, demonstrating transphosphorylation of FLT3 WT proteins. Abundant phosphorylation of the mature protein indicates that complex formation and mutual phosphorylation takes predominantly place at the cytosolic membrane. Next, co-synthesis of FLT3 ITD-GPAC and FLT3 ITD-KA-HA proteins was used to validate transphosphorylation of the FLT3 ITD molecules. As expected, the

exclusive expression of FLT3 ITD-KA-HA did not reveal any phosphorylation. In contrast, FLT3 ITD-KA-HA was phosphorylated by FLT3 ITD-GPAC (**Fig. 2B**). Since the FLT3 ITD variants of the proteins are predominantly retained intracellular in their immature high-mannose forms (14), the immature 130 kDa protein was preferably phosphorylated. Thus, it can be concluded, that transphosphorylation occurs in the biogenesis route. Important, the combinatory expression of FLT3 ITD-GPAC with FLT3 WT-KA-HA encoding gene revealed the transphosphorylation of the kinase inactive WT protein by its FLT3 ITD counterpart (**Fig. 2C**). Here it is worthwhile to mention that phosphorylation of immature high mannose 130 kDa FLT3 WT as well as complex glycosylated form of the FLT3 WT of 150 kDa could be detected. This observation indicates that FLT3 ITD - FLT3 WT complex formation and transphosphorylation apparently occurs at the ER/ Golgi as well as the cytosolic membrane.

Taken together, our data reveal mutual complex formation and transphosphorylation of FLT3 proteins with different mutational status. Despite the use of the artificial HEK293T model cell system, the maturation of the FLT3 WT as well as FLT3 ITD is similar to hematopoietic cells systems. Thus, it can be expected that it reflects the situation in leukemic cells. The intermolecular phosphorylation of oncogenic FLT3 ITD to its WT counterpart would have severe consequences for the oncogenic cell transformation. Thus, it is likely that frequently occurring monoallelic FLT3 ITD mutations might activate the entire population of cellular FLT3 molecules. Thus, beside the known consequences of the allelic ratio of FLT3 ITD to FLT3 WT the overall expression of FLT3 proteins might affect the oncogenicity of the transformed cells. In addition, the above demonstrated transphosphorylation of FLT3 WT by FLT3 ITD is a possible explanation of the observed attenuation of FLT3 inhibitors in cells co-expressing FLT3 WT and FLT3 ITD shown by Chen and co-workers (13), which consequently has important clinical implications for the design of AML therapeutic strategies.

The study respects the ethical rules of Germany.

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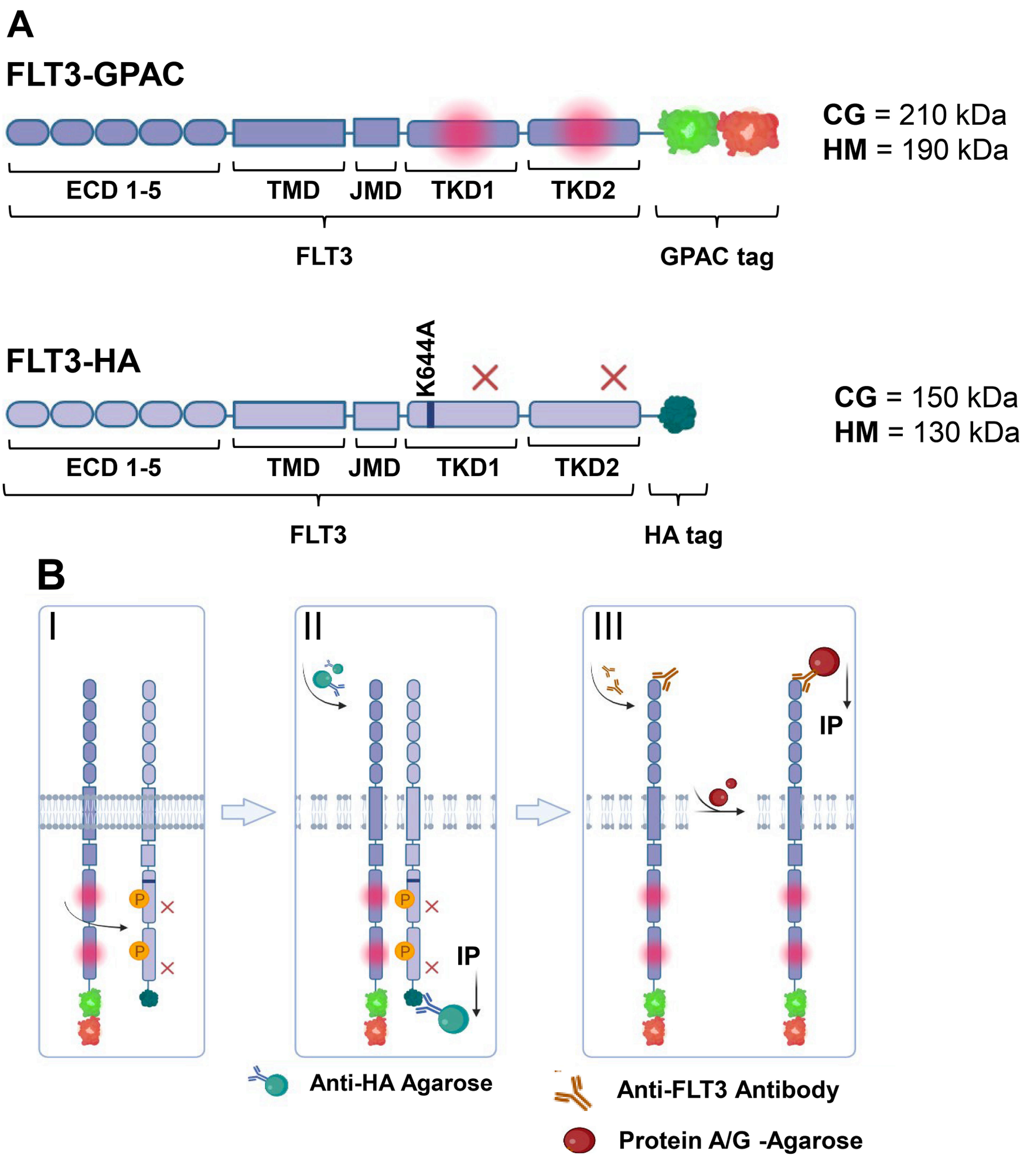
FIGURES

Fig. 1. Schematic view of FLT3 constructs and immunoprecipitation strategy. (A) Extra cellular domain (ECD), trans-membrane domain (TMD), juxta-membrane domain (JMD), complex glycosylated form of the protein (CG), high mannose form of the protein (HM) are presented. FLT3 genes were fused with the epitope regions encoding GPAC (bivalent GFP-mCherry) (top) or HA (down). HA-tagged proteins were synthesized as kinase dead K644A mutants. Red area over tyrosine kinase domain 1 (TKD1) and 2 (TKD2) indicate kinase activity of these regions, while red X above TKD1 and TKD2 represent absence of activity. **B)** Illustration of the two-step immunoprecipitation strategy. In cells co-expressing FLT3-GPAC and FLT3-KA-HA proteins transphosphorylation of FLT3-KA-HA occurs **(I).** After cell lysis immunoprecipitation of FLT3-KA-HA proteins was done using anti-HA agarose beads **(II).** Subsequently, remaining FLT3-GPAC proteins were immunoprecipitated with FLT3 antibodies and Protein A/G beads **(III).**

Fig. 2. Trans-autophosphorylation of FLT3 proteins. Immunoblots show immunoprecipitated protein of exclusive (GPAC-tagged FLT3 proteins on the left and FLT3-KA-HA on the right) and coexpressed FLT3 proteins (middle) as indicated. The FLT3 molecules are present as immature ER/Golgi-localized FLT3 form is the high mannose (HM) form of the protein and as mature, membrane localized complex glycosylated form (CG) form. The blue molecular weight standard band (M) is 180 kDa in size. Demonstrated are expression of FLT3 WT-GPAC and FLT3 WT-KA-HA (**A**), FLT3 ITD-GPAC and FLT3 ITD-KA-HA (**B**) and FLT3 ITD-GPAC and FLT3 WT-KA-HA (**C**)**.** FLT3 WT expressing samples (A) were preincubated with FLT3 ligand (200 µg/ml). Shown are immunoblots probed with phosphorylation-specific FLT3 Tyr_{591} antibodies (top) and pan-FLT3 specific S-18 antibodies (bottom).x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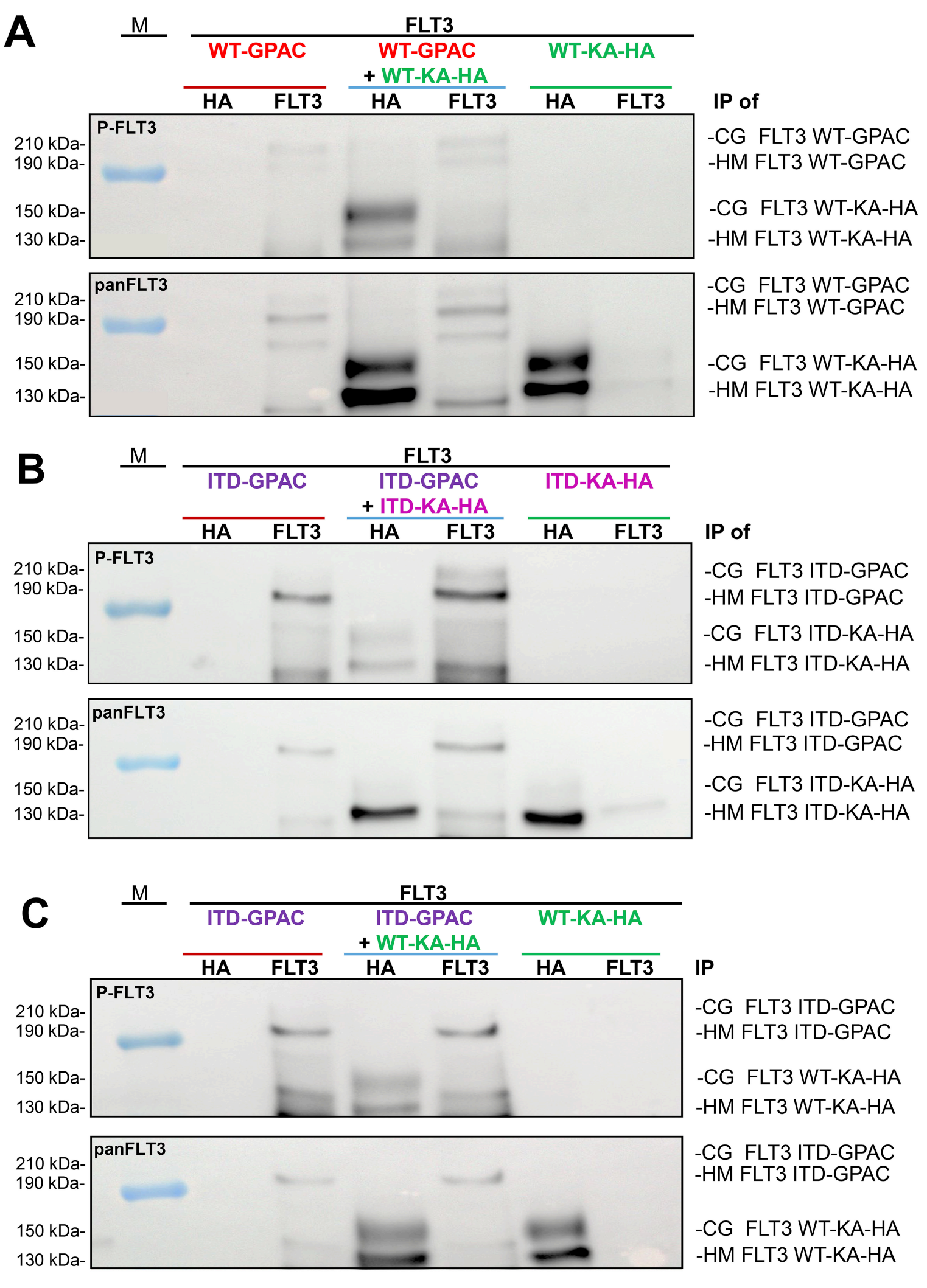
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Supplementary table 1: Materials used in the experimental approach.

Reagent	Manufacturer, Cat. No.	Preparation	Usage
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, 6429	Suplemented with: - 10% fetal calf serum, - 1% penicillin–streptomycin, - 1 % sodium pyruvate	Cell cultivation
Fetal calf serum	Anprotec, AC-SM-0190	-	
Penicillin–streptomycin	Sigma Aldrich, P0781-100ML	-	
Sodium pyruvate	Sigma-Aldrich, S8636	-	
Polyethyleneimine (PEI MAX)	KyforaBio, Polysciences, 24765-100	1 mg/ml stock solution, pH=7.0;	Transfection of cells
FLT3 ligand	ImmunoTools, Cat. No. 11343307	100 mg/ml stock solution; Final treatment concentration 200 ng/mL.	Stimulation of cells expressing FLT3 WT proteins
Trypsin	Gibco, ThermoFisher, 25300-054	-	Harvestion of cells
Lysis buffer	In house preparation	50 mM HEPES pH 7.4, 150 mM NaCl, 0.5 % NP-40, 1 mM EDTA; supplemented with protease and phosphatase inhibitor cocktails.	Cell lysis, Wash buffer (without or with 0.5 % NP40)
Protease inhibitor cocktail (cOmplete Tablets, EDTA-free, EASYpack)	Roche, 04693132001	25x stock solution in lysis buffer; Used as 1x solution in lysis buffer.	
Phosphatase inhibitor cocktail (phosSTOP, EASYpack)	Roche, 0490683700	10x stock solution in lysis buffer; Used as 1x solution in lysis buffer.	
Pierce™ Anti-HA Epitope Tag Antibody Agarose conjugate	ThermoFisher, 26181	20 µl per sample; Pre-washed three times using lysis buffer containing 0.1 % NP-40.	Immunoprecipitation of HA-tagged proteins
Anti-huFLT3 S18 antibody	Kindly gifted by Prof. Lars Rönnstrand	1 µg used in IP; 1:10000 Dilution in blocking buffer for Western blot	Immunoprecipitation of GPAC-tagged proteins, Detection of total FLT3 proteins
Protein A/G PLUS-Agarose	Santa Cruz Biotechnology, sc-2003	20 µl per sample; Pre-washed three times using lysis buffer containing 0.1 % NP-40.	Immunoprecipitation of GPAC-tagged proteins
5.4 % Polyacrylamide gels	In house preparation	Stacking gel: - Water 57.20 %, - 30 % Acrylamide 16.67 %, - Buffer 25.03 %,	SDS–PAGE separation of proteins

		- 10 % APS (Ammonium persulphate solution) 1 % TEMED (N´N´N´N´-Tetraacetylethylenediamine) 0.1 % Separation gel: - Water 55.72 %, - 30 % Acrylamide 18.20 %, - Buffer 24.98 %, - 10 % APS 1 % - TEMED 0.1 %	
Nitrocellulose membranes (AmershamTMProtrantmPremium 0,2 µm NC)	Cytiva, 10600004	Pre-socked in transfer buffer	Transfer of separated proteins
Tris-buffered saline (TBST)	In house preparation	10x stock buffer: - 200 mM Tris - 1.37 M NaCl - 1% Tween 20	Membrane wash buffer
Blocking buffer	In house preparation	1 % Bovine serum albumin in TBST	Blocking of membranes, Preparation of antibody dilutions
Phospho-FLT3 [Tyr_{591}] [54H1] mouse monoclonal antibody	Cell signaling Technology, 3466	1:1 000 Dilution in blocking buffer	Detection of phosphorylated FLT3 proteins
Anti-mouse IgG [H+L], peroxidase-labelled antibody	SeraCare, 5220-0341	1:10000 Dilution in blocking buffer	Detection of Phospho-FLT3 [Tyr_{591}] antibody
Enhanced chemiluminescence reagents (PURCEL™ Dura)	Vilber, PU4400500	-	Visualisation of immunoreactive bands
Restore™ PLUS Western Blot Stripping Buffer	Thermo Scientific, 46430	-	Stripping of membranes
Anti-rabbit IgG [H+L], peroxidase-labelled antibody	SeraCare; 5220-0336	1:10000 Dilution in blocking buffer	Detection of Anti-huFLT3 S18 antibody proteins