Elucidation of molecular basis of osteolytic bone lesions in advanced multiple myeloma

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Abstract

Osteolytic bone lesion is a major cause of lower quality of life and poor prognosis in patients with multiple myeloma (MM), but molecular pathogenesis of the osteolytic process in MM remains elusive. Fms-like tyrosine kinase 3 ligand (FLT3L) was reported to be elevated in bone marrow (BM) and blood of patients with advanced MM who often show osteolysis. Here, we investigated a functional link of FLT3L to osteolytic process in MM. We recruited 86, 306, and 52 patients with MM, acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL), respectively. FLT3L levels of patients with hematologic malignancies were measured in BM-derived plasma and found to be significantly higher in MM than in AML or ALL, which rarely show osteolysis. FLT3L levels were further elevated in MM patients with bone lesion compared with patients without bone lesion. *In vitro* cell-based assays showed that the administration of FLT3L to HEK293T, HeLa, and U2OS cells led to an increase in the DKK1 transcript level through STAT3 phosphorylation at tyrosine 705. WNT reporter assay showed that FLT3L treatment reduced WNT signaling and nuclear translocation of β-catenin. These results collectively show that the FLT3L-STAT3-DKK1 pathway inhibits WNT signaling-mediated bone formation in MM, which can cause osteolytic bone lesion. Finally, transcriptomic profiles revealed that *FLT3L* and *DKK1* were predominantly elevated in the hyperdiploidy subtype of MM. Taken together, FLT3L can serve as a promising biomarker for predicting osteolytic bone lesion and also a potential therapeutic target to prohibit the progression of the osteolytic process in MM with hyperdiploidy.

Supplementary Information for

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Supplementary Methods
Supplementary Figures S1-S6
Supplementary Table S1

SUPPLEMENTARY METHODS

Nuclear and cytoplasmic fractionation

Cells harvested in ice-cold PBS were fractionated by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). In brief, the cell pellet fully suspended by ice-cold CER I was incubated on ice for 10 minutes, and ice-cold CER II reagent was added into the tube. After repeating vortexing and incubation on ice twice, the tube was centrifuged, and the supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube. The remaining pellet was suspended in ice-cold NER for isolating the nucleus. The suspended pellet was centrifuged, and the supernatant (nuclear extract) was then transferred to a new pre-chilled tube.

Western blotting

Plasmid DNA was transfected into HEK293T cells, which were seeded with 70% confluency using TransIT®-LT1 reagent (Mirus, Madison, WI, USA). Cells treated with recombinant protein or inhibitor for 2~4 days were harvested, lysed by lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, and 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Roche) and then quantified by Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) and NanoDrop (Thermo Fisher Scientific, Waltham, MA). Samples were eluted in a protein sample buffer (Elpis Biotech, Daejeon, Korea) and boiled for 5 minutes. Proteins were separated by 8~15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were blotted onto polyvinylidene difluoride (PVDF) transfer membrane with 0.45 μm pore size (Merck Millipore, Burlington, MA). Blots were blocked in 1X PBS with 0.1% Tween-20 (Sigma) containing 5% Difco™ Skim Milk (BD Biosciences, East Rutherford, NJ) for 1 hour at room temperature and incubated with primary antibodies against phosphor-STAT3 (Cell Signaling Technology, Danvers, MA), STAT3 (Cell Signaling Technology), DKK1 (Abcam, Cambridge, UK), β-catenin (BD biosciences), GAPDH (Santa Cruz Biotechnology, Dallas, TX), α-Actinin (Santa Cruz Biotechnology), α-Tubulin (AbFrontier, Seoul, Korea), and Lamin A/C (Santa Cruz

Biotechnology). After blots were washed in 1X PBS-Tween20 buffer, the blots were incubated in anti-mouse secondary (Jackson ImmunoResearch, West Grove, PA) or anti-rabbit secondary (Jackson ImmunoResearch) for 2 hours at room temperature. Bands were detected by enhanced chemiluminescence solution (Bio-Rad) using ChemiDoc System (Bio-Rad).

Luciferase reporter assay

HEK293T cells were plated in the Falcon® 96-well white flat bottom tissue culture-treated microtest assay microplate (CORNING, Corning, NY, USA). In each well, 5,000 cells were plated in 100 µL 10% DMEM media and cultured for 24 hours. The cells were transfected with pcDNA3.1-empty vector or pcDNA3.1-DKK1, M50 Super 8x TOPFlash plasmid (obtained from addgene plasmid #12456), and pNL1.1.TK internal control vector for the assay, using calcium phosphate transfection Kit (Invitrogen, Carlsbad, CA, USA). Using Nano-Glo® Dual-Luciferase® Reporter Assay Kit (Promega), luciferase activity was measured in triplicate according to the manufacturer's instructions. The firefly luciferase activity was normalized against the NanoLuc® luciferase activity.

Murine BM-derived macrophage (BMM) isolation and osteoclast differentiation

For preparing macrophage cultures, Femur and tibia from hind legs of C57BL/6 male mouse (age: 4~6 weeks) were obtained, and their epiphyses were cut off. BM was flushed out with α-MEM media (Gibco) by using syringe. Pellet of BM cells collected by centrifugation was lysed by Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma), the remaining cells were spun downed, and cells resuspended with α-MEM media supplemented with 10% FBS, 100U/mL pen-strep and 30 ng/mL M-CSF (R&D SYSTEMS) were cultured for 3~4 days following filtration by strainer. For osteoclast differentiation, adherent BMM was trypsinized and cultured in triplicates in 48-well plate (5 x 10^4 cells/well) with α-MEM media supplemented with 10% FBS, 100U/mL pen-strep, 30 ng/mL M-CSF and 100 ng/mL RANKL (R&D SYSTEMS) for 3~5 days. Media were changed every two day.

Tartrate-resistant acid phosphatase (TRAP) staining and activity assay

Osteoclast differentiation capacity of BMM was analyzed by TRAP staining and absorbance of reactant. For TRAP staining, osteoclast cells cultured in 48-well plate were stained by Leukocyte Acid Phosphatase kit (Sigma). According to the manufacturer's protocol, cells were washed by PBS and fixed by fixative solution (Citrate solution, acetone, and 37% formaldehyde) for 5 minutes. Fixed cells were washed three times by using deionized water prewarmed to 37℃. TRAP staining solution (Fast Garnet GBC, Sodium Nitrite, Naphtol AS-BI Phosphate, Acetate, and Tartrate solution) was added to each well and incubated at 37℃ in dark. After 1 hour, cells were rinsed three times in deionized water and dried at room temperature. TRAP-positive multinucleated cells were stained red. To measure TRAP activity (TaKaRa Bio, San Jose, CA), differentiated cells were washed by PBS and lysed with extraction solution (physiological saline including 1% NP-40). Substrate solution (pNPP substrate and Acid phosphatase buffer) was added to lysate of each well and incubated at 37℃ for 1 hour. Finally, stop solution (0.5 N NaOH) was added to each well, and the absorbance was measured at 405 nm using a GloMax® Discover System (Promega, Madison, WI).

Osteoblast differentiation

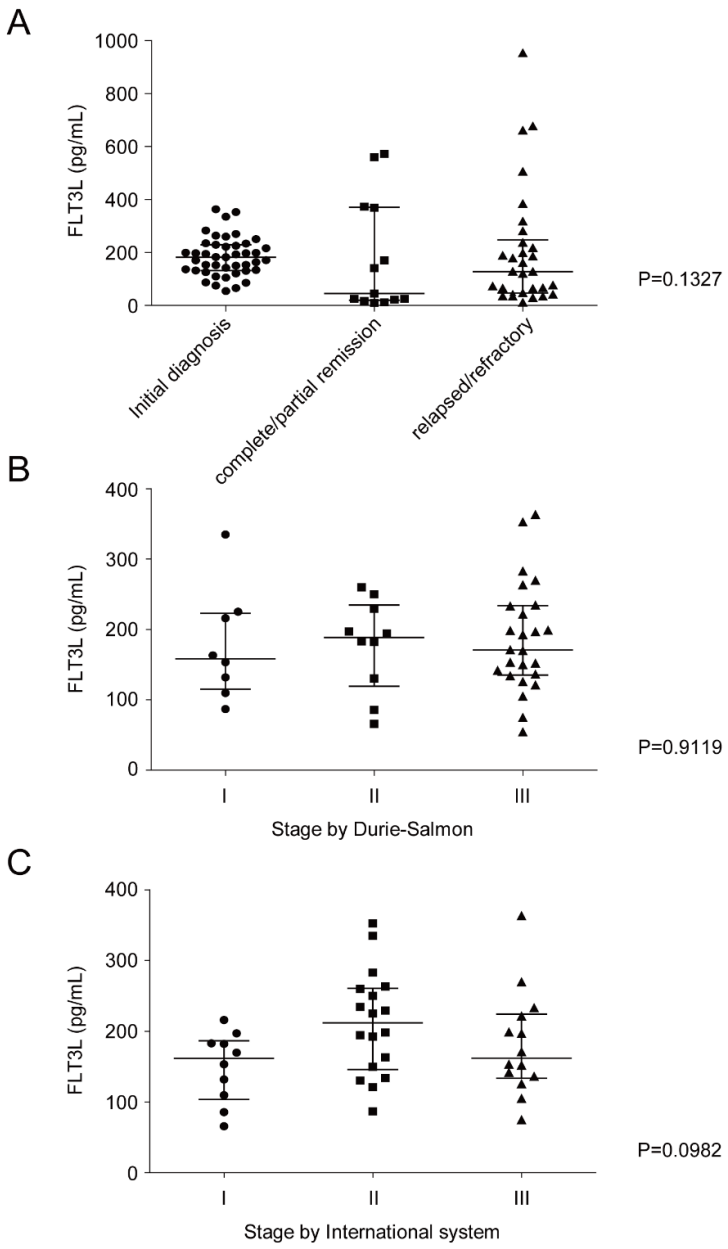
Mouse osteoblastic cell line (MC3T3-E1) were seeded into 24-well cell culture plates at a density of $3x10^4$ cells/well and then cultured in α-MEM (GIBCO, Cat#A10490) supplemented with 10% FBS, 1% penicillin-streptomycin at 37℃ with 5% CO_2. To induce differentiation, cells with 80~90% confluence were treated with recombinant human FLT3L, BMP2, or DKK1 and maintained with replacement of fresh medium every 2~3 days for 7 days.

Alkaline phosphatase (ALP) staining and activity

For the detection of alkaline phosphatase, cells were initially cultured with osteogenic differentiation media for 2 or 3 days. Subsequently, the cells were gently washed with PBS and then fixed with 4% paraformaldehyde. After fixation, the cells were rinsed with washing buffer (0.05% Tween 20 in PBS) and then treated with a substrate solution prepared by dissolving one BCIP/NBT tablet (Sigma) in 10 ml of distilled water. For staining, the cells were incubated at room temperature in the dark for 10 minutes, with monitoring of the staining progress every 2-3 minutes. The substrate solution was carefully aspirated, and the cells were rinsed with washing buffer. For ALP activity assessment, cultured cells were washed with PBS and subsequently lysed using a cold alkaline phosphatase reaction buffer (composed of 1M Diethanolamine and 0.5 mM Magnesium Chloride at pH 9.8, Sigma). Fresh reaction buffer was pipetted into 96-well plate, and to each well, a 0.67 M *p*-Nitrophenyl Phosphate (pNPP) solution (Sigma) was added, followed by incubated for 30 minutes at 37℃. After incubation, the lysates were added to each well, and the reaction was immediately followed by monitoring absorbance at 405 nm. Total protein content was measured using Pierce™ BCA protein assay kit (Thermo Fisher Scientific) and read at 562 nm using a SpectraMax® instrument (Molecular Devices). The enzymatic ALP activity was normalized to the protein content of the samples.

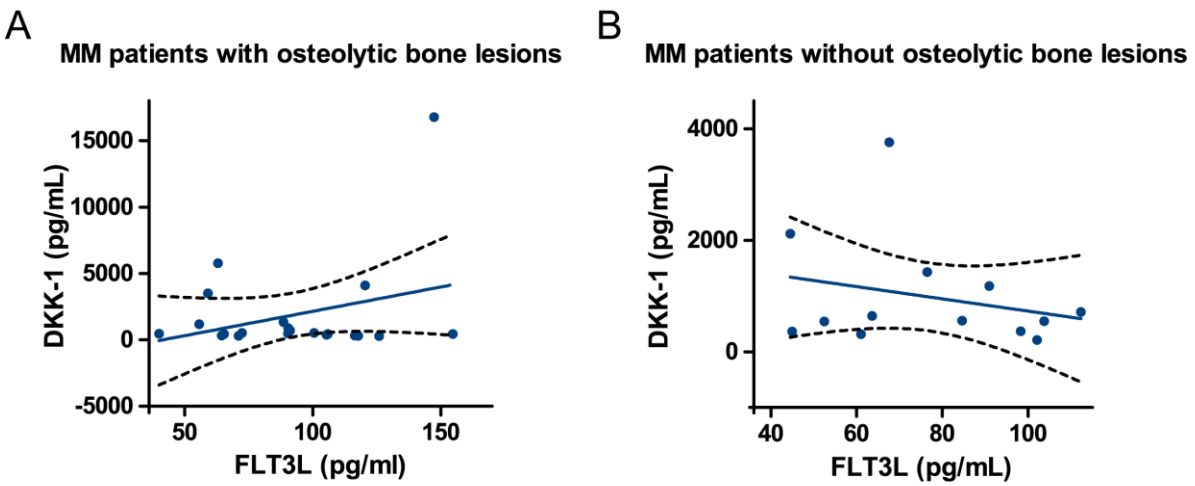
SUPPLEMENTARY FIGURES

Supplementary Figure S1



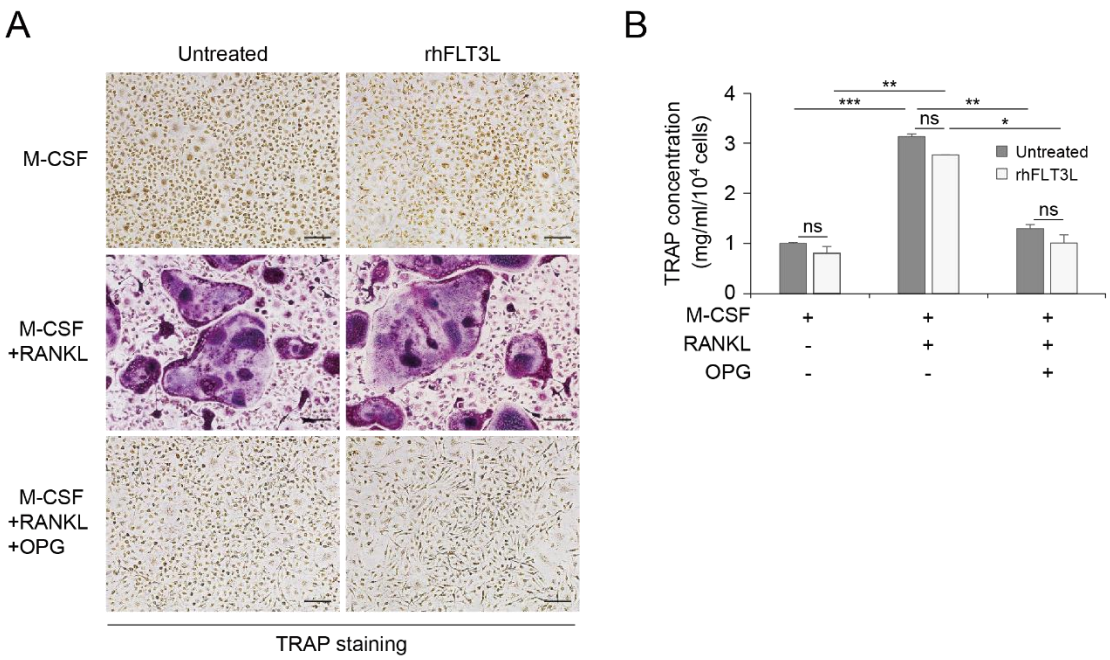
Supplementary Figure S1. The plasma level of FLT3L in bone marrow of multiple myeloma patients was significantly associated neither with (A) the disease status of initial diagnosis (n=43), complete or partial remission (n=13), and relapsed or refractory disease (n=30) (*P*=0.13), nor with the stage by (B) Durie-Salmon and (C) International Staging System at the time of initial diagnosis (*P*=0.91 and 0.098, respectively)

Supplementary Figure S2



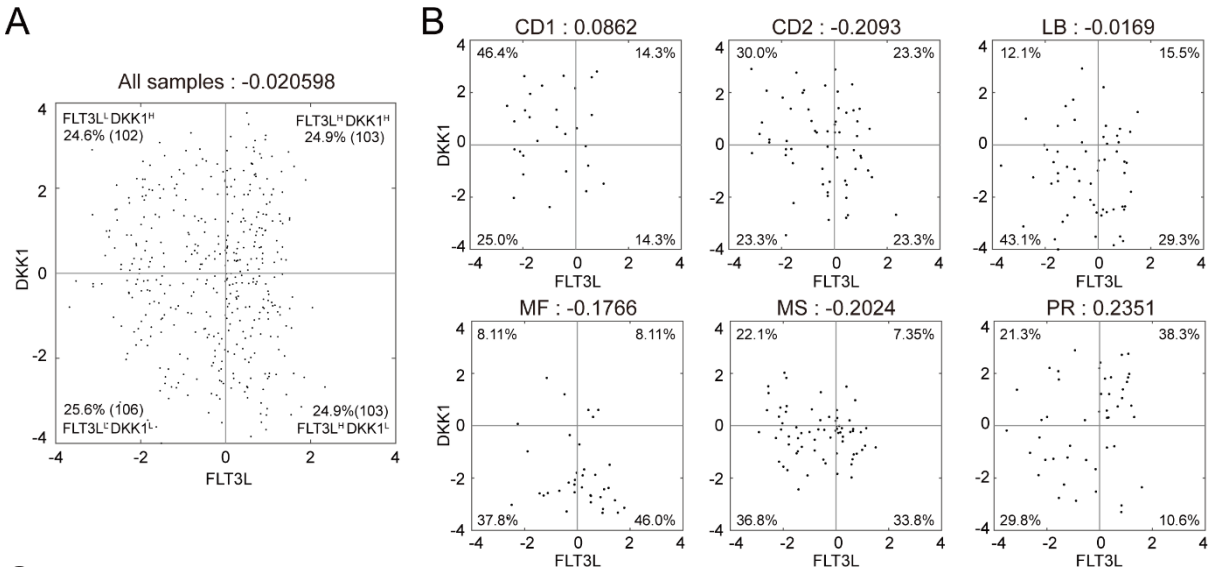
Supplementary Figure S2. The DKK1 level was determined by ELISA in a subgroup of MM patients with (A) or without (B) osteolytic bone lesions (n=35). FLT3L and DKK1 levels tends to show a positive correlation in MM patients with osteolytic bone lesion (Adj R-squared = 0.0922, p = 0.1809), but a negative correlation in MM patients without osteolytic bone lesion (Adj R-squared = - 0.0183)

Supplementary Figure S3



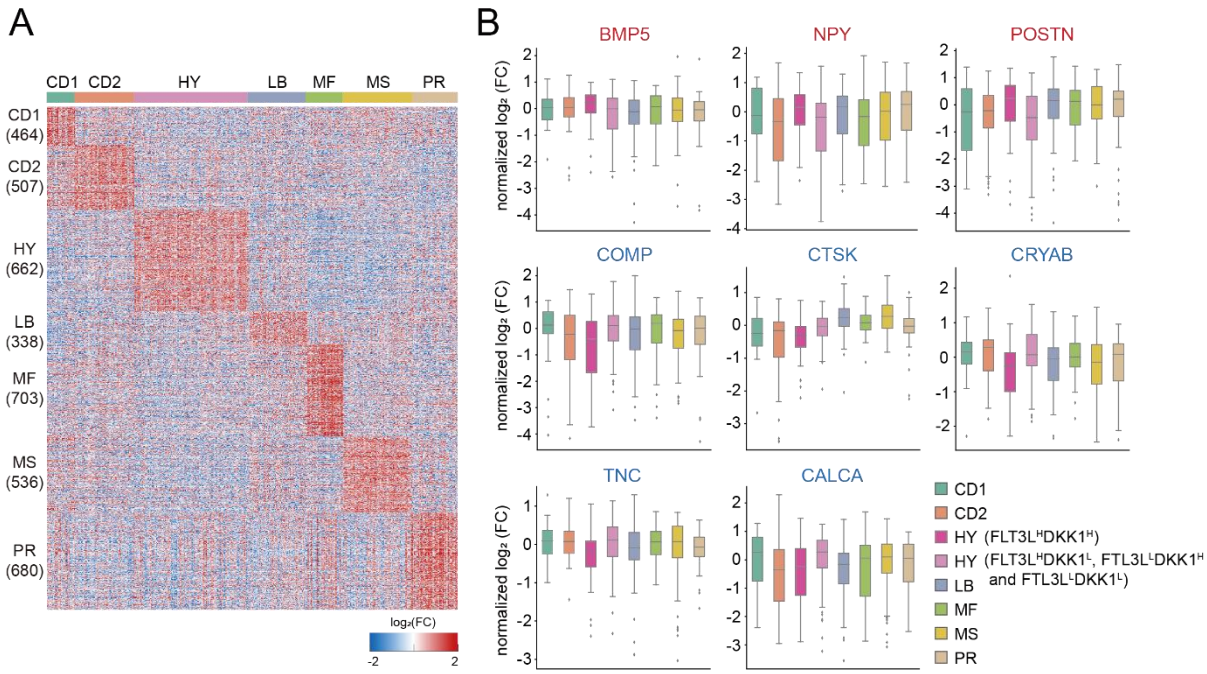
Supplementary Figure S3. (A) Representative images of TRAP-positive osteoclasts induced by M-CSF (30 ng/mL) and RANKL (100 ng/mL) to differentiate mouse bone marrow cells treated with and without rhFLT3L (40 ng/mL) for 3 days. 100 ng/mL osteoprotegerin (OPG) used as a RANKL inhibitor was treated into bone marrow cells. Scale bar in each picture means 20 μm. (B) TRAP activity was determined by measuring absorption at 405 nm (n=4). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns, not significant (*P*>0.05). Statistics was performed by two-way ANOVA with Tukey's multiple comparisons test. Error bars indicate the standard deviation of independent four replicates.

Supplementary Figure S4



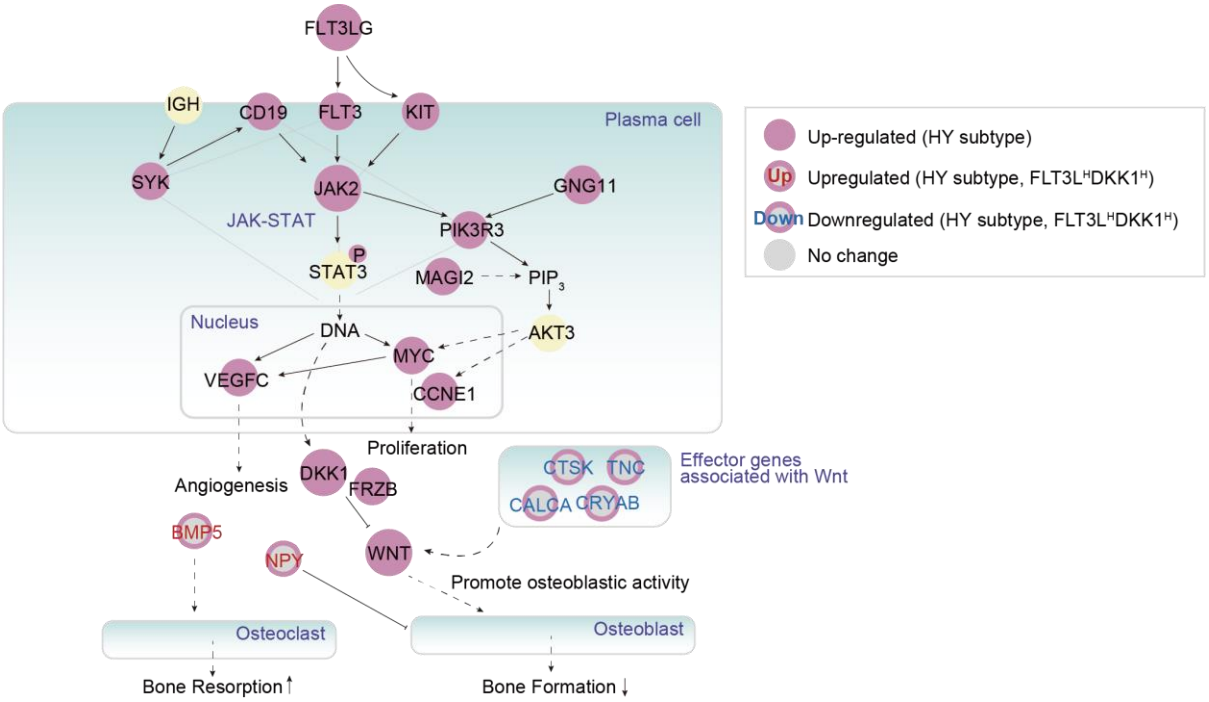
Supplementary Figure S4. (A) Scatterplot showing log_2-fold-changes of mRNA expression levels between FLT3L (x-axis) and DKK1 (y-axis) in all 414 patients with MM. Log_2-fold-changes were computed with respect to the median mRNA expression value in all sample. Spearman's correlation between FLT3L and DKK1 is displayed, and the percentage of patients in each quadrant is also shown. (B) Scatterplot showing log_2-fold-changes of mRNA expression levels between FLT3L (x-axis) and DKK1 (y-axis) in seven subtypes of MM (CD1, CD2, HY, LB, MF, MS and PR). Spearman's correlation between FLT3L and DKK1 and the percentage of patients in each quadrant are displayed in each subtype. The median expression value for each gene was used to define high expression of the gene.

Supplementary Figure S5



Supplementary Figure S5. (A) Heat map showing log_2-fold-changes of signature genes predominantly upregulated in each subtype. The color bar represents the gradient of log_2-fold-changes of mRNA expression levels in the samples with respect to the median mRNA expression levels. Numbers in parenthesis indicate the numbers of signature genes for each subtype. (B) Boxplots showing distributions of expression levels of the indicated genes in the six subtypes, FLT3L^{H}DKK1^H HY subgroup and the other HY subgroups. Q1-3 in each distribution are represented by the bottom, median line, the top of the box, respectively, and whisker bar denotes the distribution of expression levels below Q1 and Q3.

Supplementary Figure S6



Supplementary Figure S6. A network model describing interactions among the upregulated genes involved in the pathways selected in Figure 4D. Node color represents the signature genes for the HY subtype versus others. Gray nodes indicate the non-upregulated genes added in the network to increase connections among the genes. Solid and dashed edges represent direct and indirect activation (arrow) or repression (suppression symbol), respectively. Gray lines indicate protein-protein interactions. Thich gray lines denote the plasma and nuclear membranes.

Supplementary Table S1

Table S1. Clinical factors related to FLT3L level (treatment-naive patients with MM, N=43)

		Multivariate		
Variables		HR	95% CI	P
Age	+1 years	0.925	0.837-1.022	0.127
Sex	Female	-	-	-
	Male	0.241	0.017-3.416	0.293
BM plasma cell	+1%	1.116	1.011-1.232	**0.029**
Serum M-protein	+1 g/dL	0.499	0.235-1.060	0.071
Osteolytic lesion	No	-	-	-
	Single lesion	39.878	0.707-2249.678	0.073
	Multiple lesion	115.423	0.690-19306.497	0.069
Karyotype	Normal	-	-	-
	Hypodiploidy	0.112	0.002-4.982	0.258
	Hyperdiploidy	245.341	1.150-52319.514	**0.044**
Stage	I	-	-	-
	II	4.807	0.049-467.796	0.501
	III	0.231	0.004-12.835	0.587