Research Article: Translational Cancer Mechanisms and Therapy

EGFL7 Antagonizes NOTCH Signaling and Represents a Novel Therapeutic Target in Acute Myeloid Leukemia

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Running title: Targeting EGFL7 in AML

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STATEMENT OF TRANSLATIONAL RELEVANCE

Our previous work showed that EGFL7 is overexpressed in AML and associated with poor outcome. Here, we further dissect the possible mechanisms underlying EGFL7 leukemogenesis. We found that binding of EGFL7 to NOTCH receptors antagonizes canonical NOTCH ligand binding, resulting in down regulation of NOTCH target genes. Although NOTCH receptors are expressed at significant levels in AML blasts, NOTCH signaling is silenced. Re-activation of NOTCH signaling results in blast differentiation and apoptosis. More importantly, inhibiting EGFL7 using an anti-EGFL7 blocking antibody (Parsatuzumab) results in re-activation of the NOTCH pathway resulting in blast differentiation, apoptosis, and prolonged survival in three independent mouse model. Since, Parsatuzumab has already been shown to be safe in Phase I and II clinical trials for solid tumors, it has the potential to be used alone or in combination with current FDA-approved drugs for AML.

ABSTRACT

Purpose: Epidermal growth factor-like domain 7 (EGFL7) is a secreted protein and recently has been shown to play an important role in acute myeloid leukemia (AML); however, the underlying mechanism by which EGFL7 promotes leukemogenesis is largely unknown.

Experimental Design: Using an antibody interaction-array we measured the ability of EGFL7 to bind directly ~400 proteins expressed by primary AML blasts. Primary patient samples were stimulated *in vitro* with recombinant EGFL7 (rEGFL7) or anti-EGFL7 blocking antibody to assess alterations in downstream signaling and the ability to effect blast differentiation and survival. We treated three independent AML models with anti-EGFL7 or IgG1 control to determine whether anti-EGFL7 could prolong survival *in vivo*.

Results: We found EGFL7 significantly binds several signaling proteins important for normal and malignant hematopoiesis including NOTCH. Stimulation of AML blasts with rEGFL7 reduced NOTCH intracellular domain and NOTCH target gene expression while treatment with an anti-EGFL7 blocking antibody resulted in reactivation of NOTCH signaling, increased differentiation, and apoptosis. Competitive ligand binding assays showed rEGFL7 inhibits DELTA-like (DLL) 4-mediated NOTCH activation while anti-EGFL7 combined with DLL4 significantly increased NOTCH activation and induced apoptosis. Using three different AML mouse models, we demonstrated that *in vivo* treatment with anti-EGFL7 alone results in increased survival.

Conclusion: Our data demonstrates that EGFL7 contributes to NOTCH silencing in AML by antagonizing canonical NOTCH ligand binding. Reactivation of NOTCH signaling *in vivo* using anti-EGFL7 results in prolonged survival of leukemic mice, supporting the use of EGFL7 as a novel therapeutic target in AML.

INTRODUCTION

Epidermal growth factor-like domain 7 (EGFL7) is a secreted protein and plays an important role in angiogenesis by regulating the growth, proliferation and migration of endothelial cells (1-3). In solid tumors, it has been shown that *EGFL7* is overexpressed and associates with a more aggressive disease (4). We previously found that *EGFL7* is up-regulated in primary AML blasts and that high *EGFL7* mRNA expression correlates with shorter event-free and overall survival in AML patients. Moreover, we demonstrated that AML blasts are able to secrete EGFL7 protein and treatment of primary AML blasts with rEGFL7 *in vitro* leads to increases in leukemic blast cell growth (5). These data suggest an important clinical and biological role for EGFL7 in AML; however, the molecular mechanisms involving EGFL7-mediated leukemogenesis have not been thoroughly examined. EGFL7 has been shown to bind NOTCH receptors and can activate or antagonize canonical NOTCH signaling (2,6,7). Whether EGFL7 represses or activates NOTCH signaling in AML has not been determined.

NOTCH signaling is an evolutionarily conserved pathway and plays an important role in regulating cell-fate determination during development and maintenance of adult tissue homeostasis (8,9). The NOTCH receptor is a single transmembrane protein composed of a functional extracellular (EC), transmembrane (TM), and intracellular (IC) domain. Signaling through NOTCH requires the activation of these receptors by binding canonical NOTCH ligands such as JAG1, JAG2, and DLL 1,3,4, resulting in intracellular cleavage of NOTCH protein to the truncated form (NICD), which then translocates to the nucleus to activate gene transcription (9-11). NOTCH signaling has been shown to be important for normal hematopoiesis and stem cell function (8,12-15). Mutations in NOTCH receptors can result in NOTCH signaling hyperactivation, a well-known driver of leukemogenesis for a subset of T cell acute leukemia (8). However, NOTCH signaling

has been shown to have the opposite role in AML (16,17). Several groups have shown that NOTCH signaling is silenced in AML and that reactivation of NOTCH signaling results in blast differentiation and disease elimination (16,17). However, the mechanism underlying this NOTCH inactivation in AML has not been determined (16-18).

Here, we found that EGFL7 is able to bind many different signaling proteins important for regulating hematopoiesis including NOTCH. We confirmed these finding using co-immunoprecipitation assays and found that EGFL7 binds NOTCH receptors on primary AML blasts and cell lines. Stimulation of blasts with rEGFL7 protein results in decreases in the levels of NICD and NOTCH target genes. Conversely, blasts treated with an anti-EGFL7 blocking antibody showed reactivation of NOTCH activity resulting in increases in expression of downstream target genes, NICD levels, and apoptosis. Primary blasts treated with a γ-secretase inhibitor blocked anti-EGFL7 induced NOTCH activation, substantiating our findings that NOTCH signaling is downstream of EGFL7 activity in AML. We also found the NOTCH ligands DLL1 and DLL4-mediated activation of NOTCH signaling can be inhibited by rEGFL7, while anti-EGFL7 treatment enhanced the activation of NOTCH by these ligands. Overall, we demonstrate that EGFL7 silences NOTCH signaling in AML by antagonizing canonical NOTCH ligand binding and that anti-EGFL7 therapy represents a rational, novel targeted therapy with low toxicity.(19) Although we show biological activity as a single agent, we are also currently investigating the use of anti-EGFL7 therapy in combination with other currently approved drugs for AML in hope of a more effective, low toxic cure for patients with AML.

MATERIALS AND METHODS

Reagents and cells

Recombinant human DLL4 (Fc chimera, ab108557) and natural human IgG Fc fragment proteins (ab90285) were obtained from Abcam (Cambridge, MA, USA). Anti-human EGFL7 therapeutic antibody (Parsatumumab) was obtained from Creative Biolabs (Shirley, NY, USA). Normal human immunoglobulin (Immune Globulin, 3036917) was purchased from Grifols (Barcelona, Spain). γ-secretase inhibitor (Avagacestat [AVA], BMS-708163) was obtained from Selleckchem (Houston, TX, USA). OP9 and OP9-DLL1 mouse stromal cells were cultured in MEM alpha/GlutaMAX medium (Gibco, Thermo Fisher Scientific [Waltham, MA, USA]) supplemented with 20% fetal bovine serum (FBS).

Primary AML blasts and AML cell lines

THP-1, K-562 and Kasumi-1 cells were newly purchased from American Type Culture Collection (ATCC, Manassas, VA, USA); EOL-1 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Thawed cells were passaged not more than three times before the experiments were performed. AML cell lines were cultured in RPMI1640 (Gibco) supplemented with 10-20% FBS (Gibco). AML blasts used in the experiments were obtained from apheresis blood samples collected from patients treated at The Ohio State University (OSU) and stored in the OSU Leukemia Tissue Bank. Patients provided written informed consent, and protocols were in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards. Primary blasts from AML patients were maintained in SFEM (StemCell Technologies [Vancouver, Canada]) medium supplemented with 10% FBS and 1x StemSpan CC100 cytokine cocktail (StemCell Technologies) unless otherwise noted.

AML mouse models

All studies using animals were carried out in accordance with the OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee. All mouse experiments were unblinded. All mice used in the experiments were between 8 and 10 weeks of age. EOL-1 mouse model: Female NOD-*scid* IL2Rg^{null} (NSG) mice were transplanted with 1x10^{7} EOL-1 cells. Two weeks post-transplant mice were treated with anti-EGFL7 or IgG1 control (4 mice per group; dosage 50 mg/kg body weight intraperitoneal), 3 times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. Murine AML model: we used our previously established *Mll*$^{PTD/WT}$;*Flt3*$^{ITD/WT}$ dKI mouse model that is maintained on a pure C57Bl/6J background.[5] In the primary transplant, lethally irradiated (10 Gy) BoyJ (C57Bl/6J-CD45.1) were intravenously injected through the tail vein with 2.0x10^{6} leukemic *Mll*$^{PTD/WT}$;*Flt3*$^{ITD/WT}$ BM cells along with 5.0x10^{5} BM from a normal BoyJ (C57Bl/6J-CD45.1) control mouse. Two weeks post-transplant mice were treated with anti-EGFL7 or IgG1 control (8 mice per group; dosage 50 mg/kg body weight intraperitoneal), 2 times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. Two mice (one per group) were excluded from the study because they were identified as statistical outliers using the "Interquartile Range" method. Patient derived mouse (PDX) model: Female NOD-*scid* IL2Rg^{null} (NSG) mice were conditioned with busulfan (30mg/kg body weight, i.p.). After 24 hours, the mice were transplanted with 2x10^{6} AML cells from cytogenetically normal AML patient 5. Four weeks post-transplant mice were treated with anti-EGFL7 or IgG1 control (4 mice per group; dosage 50 mg/kg body weight intraperitoneal), 2 times per week. Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. The experiment was stopped after 12 weeks of treatment.

RNA expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using SuperScript III reagents (Invitrogen) according to the manufacturer's instruction. Quantitative Real-Time RT-PCR was performed using commercially available TaqMan Gene Expression Assay primers and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Invitrogen). The expression levels of target genes were normalized to *β-ACTIN*. Relative expression was calculated by using the comparative $2^{-\Delta\Delta Ct}$ method.

Detection of EGFL7 interacting proteins by antibody array

Potential EGFL7 interacting proteins were probed with Signal Transduction Antibody Array containing antibodies against 400 proteins from Hypomatrix (Cat. HM3000, Worcester, MA, USA) according to the manufacturer's protocol (see also Supplemental Materials). In brief, primary patients' cells ($5\text{-}10X10^6$) were lysed in 500 µl of Pierce Co-Immunoprecipitation lysis buffer supplemented with protease and phosphatase inhibitors (Roche [Basel, Switzerland]). Cell lysis was performed on ice for 20 minutes (min) and the cell lysate was sonicated for 5 seconds (s) 3 times. After centrifugation at 14,000 g at 4°C for 15 min, the cell lysate supernatant was diluted to 3 ml in lysis buffer containing 0.1% dry milk and 10 nM recombinant human EGFL7 protein (PeproTech [Rocky Hill, NJ, USA]), added to the antibody array membrane previously blocked with 5% dry milk in tris-buffered saline with 0.05% Tween 20 (TBST) buffer for 2 h, and incubated at 4°C for 2 hours on a flat shaker with slow shaking. Following washing 5 times with TBST buffer, the membrane was incubated with HRP-conjugated anti-EGFL7 antibody (Bioss [Woburn, MA, USA]) at room temperature for 2 hours and developed using ECL Select Western Blotting Detection Reagent (GE Healthcare [Little Chalfont, UK]).

Protein co-immunoprecipitation (Co-IP)

Cells (1-5X10^6) were lysed in 400 µl of Pierce Co-Immunoprecipitation lysis buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche). Cell lysis was performed on ice for 20 min and the cell lysate was sonicated for 5 s 3 times. After centrifugation with 14,000 g at 4°C for 15 min, the cell lysate supernatant was transferred to new tubes immediately and added to 50% Protein G Plus/Protein A agarose beads (EMD Millipore [Burlington, MA, USA]) with a ratio of 100 µl for a 1ml sample solution. After incubation with rotation at 4°C for 1 h, the mixture was centrifuged at 14,000g for 10 min at 4°C. The supernatant was transferred to new tubes, 2-20 µg antibody were added and incubated on a rotating shaker at 4°C overnight. The anti-NOTCH1 ((D1E11) XP Rabbit mAb #3608) and anti-NOTCH2 ((D76A6) XP Rabbit mAb #5732) antibody, were obtained from Cell Signaling Technology (Danvers, MA, USA). Both antibodies recognize the full length and NICD of NOTCH1 and NOTCH2, respectively, according to manufacturers' product descriptions. The anti-MYC ((71D10) Rabbit mAb #2278) and anti-FLAG (DYKDDDDK) ((D6W5B) Rabbit mAb #2278) antibodies were obtained from Cell Signaling Technology. After centrifugation, the supernatant was incubated with 50 µl Protein G Plus/Protein A agarose beads at 4°C with rotation for 2 h. The agarose beads were washed 5 times with ice-cold Pierce Co-Immunoprecipitation lysis buffer. Then 20 µl 2x Laemmli sample buffer was added, boiled for 5 min and subjected to Western blotting. The corresponding normal antibodies were applied as negative controls for Co-IP.

Western blot analysis

Cells were lysed in 100 µl of Pierce RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Roche) on ice for 20 min. After centrifuging at

14,000 g for 15 min at 4°C, the supernatant was transferred into a new tube, mixed with 20 µl of 5x Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 min. Equal volume of samples (10-30 µl) were separated in precast SDS gel (Bio-Rad [Hercules, CA, USA]) and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked by freshly prepared 5% dry milk made in TBST buffer (Bio-Rad) at room temperature for 1 hours and then incubated overnight at 4°C in a primary antibody. It was then washed 3 times with TBST buffer and incubated with secondary HRP-conjugated antibody at room temperature for 1 h. Immunoreactive bands were detected using ECL Select Western Blotting Detection Reagent (GE Healthcare). Mouse anti-EGFL7 (LS-C153302) and rat anti-EGFL7 (LS-C40134) antibodies were purchased from LifeSpan BioSciences (Seattle, WA, USA); goat anti-EGFL7 (sc-34116) and rabbit anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); rabbit anti-NOTCH1 ((D1E11) XP Rabbit mAb #3608), NOTCH2 ((D76A6) XP Rabbit mAb #5732), NICD ((D3B8) Rabbit mAb #4147), β-TUBULIN ((9F3) Rabbit mAb #2128), β-ACTIN ((13E5) Rabbit mAb #4970) and HES1 ((D6P2U) XP Rabbit mAb #11988) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The NICD antibody detects only the NICD after cleavage of NOTCH1 and NOTCH2 proteins. HRP-conjugated sheep anti-mouse, goat anti-rat, donkey anti-rabbit, and donkey anti-goat secondary antibodies were bought from GE Healthcare.

Cell transfection

Transfection of K562 cells was conducted by Lonza transfection kit V following (Lonza Group Ltd, Basel, Switzerland) manufacturer's procedure. Briefly, 2.5ug of an EGFL7 (FALG-DDK-tagged) pCMV3-SP-N-FLAG vector (HG11979-NF) from Sinobiological (Wayne, PA, USA) and 2.5ug of a NOTCH2 (Myc-DDK-tagged) pCMV6-Entry vector

(RC215107) form OriGene (Rockville, MD, USA) were co-transfected. Cells were cultured for 48 hours before Co-IP was performed.

Cell apoptosis and differentiation

For the evaluation of apoptosis, cells were stained with Annexin V and 7-AAD as previously described(5,20). For cell differentiation analysis, cells were stained with an anti-CD11B or anti-CD14 antibody (BD Biosciences, Franklin Lakes, NJ, USA) in the presence of DAPI and analyzed by flow cytometry. Only DAPI negative (viable) cells were included in the differentiation analyses.

Statistical analysis

Two-tailed student's *t* tests were performed using GraphPad Prism version 7 (Graphpad Software, San Diego, CA) to analyze *in vitro* and *in vivo* experiments unless otherwise noted. *P* values <0.05 were considered significant. Overall survival for the mouse AML xenograft model was calculated using the Kaplan–Meier method, and survival curves were compared by the log-rank test.

RESULTS

EGFL7 interacts with NOTCH receptors in AML cells

Our previous studies demonstrate that *EGFL7* is upregulated in primary AML blasts and that high *EGFL7* mRNA expression correlates with poor outcome in AML patients (5). However, the mechanism by which EGFL7 contributes to leukemogenesis is unknown. In order to obtain insights about potential mechanisms by which EGLF7 stimulates AML blast growth, we identified potential EGFL7 interacting proteins. Since other members of the EGFL family of proteins are known to have numerous potential binding partners (2,21-23), we took an unbiased high-throughput approach by performing antibody interaction arrays. These arrays have 400 antibodies hybridized to a membrane. Protein lysates from AML blasts were first incubated with rEGFL7 protein, followed by incubation with the antibody array membranes. Then using an anti-EGFL7-HRP or anti-GST-HRP control antibody (**Figure 1A**), the membranes were developed and densitometry of the individual spots were determined for both the anti-EGFL7 and anti-GST-HRP control blots. Array data were then log2 transformed and normalized by subtracting the mean of the background. Subsequently, a linear mixed model was used for analysis to compare the protein level between anti-EGFL7 antibody array and anti-GST antibody array. Using this strategy, we found that NOTCH1 was bound significantly to EGFL7 in all 3 samples (P=1.11x10^{-06}).

Next, we validated our screening results by assessing direct binding between EGFL7 and NOTCH receptors in both an AML cell line (THP-1) and primary AML blasts. Using the THP-1 (**Figure S1**) cell line that expresses EGFL7 and NOTCH receptors but exhibits dampened NOTCH activation (16,17), we performed Co-IP assays using an anti-EGFL7 antibody to pull down EGFL7 containing protein complexes. We then performed western blotting to determine protein interactions using anti-NOTCH1 and 2 antibodies. Conversely, when we performed the pull-down using anti-NOTCH1 or

NOTCH2 antibody, we found that both NOTCH1 and NOTCH2 could be co-immunoprecipitated as part of a complex with EGFL7 (**Figure 1B**). These results were also confirmed using primary AML patient blasts (**Figure 1B**). We further validated our results by transfecting K562 cells with EGFL7-FLAG and NOTCH2-MYC tagged expression plasmids. Co-IP experiments were then performed using a MYC-specific antibody to pull down NOTCH2, and subsequent western blotting using an EGFL7-specific antibody. These experiments validate the interaction between the NOTCH (MYC-tagged) and EGFL7 protein. Concomitantly, using a FLAG antibody to pull down EGFL7, we found NOTCH2 to be co-immunoprecipitated with EGFL7. (**Figure 1C**). Altogether these data demonstrate the direct interaction of EGFL7 with NOTCH receptors on AML cell lines and primary AML blasts.

EGFL7 suppresses NOTCH signaling in AML cells

Previous studies suggest that the interaction of EGFL7 with NOTCH receptors may lead to inhibition of NOTCH signaling (6,24,25). To determine whether EGFL7 binding of NOTCH results in alterations of NOTCH signaling in AML patient blasts we examined the levels of the NICD produced in response to rEGFL7 stimulation of primary AML patient samples. We found that although the NICD1 antibody from Cell Signalling (D3B8) was specific for the cleaved/activated form of NOTCH (i.e. NICD), we found that it was able to recognize both NICD from NOTCH1 and NOTCH2 (**Figure S2**). Since AML blasts express both NOTCH1 and NOTCH2, we provide the endogenous *NOTCH1* and *NOTCH2* expression levels for each patient in Supplemental Materials **Figure S3** and **S4**. Stimulation with rEGFL7 results in significant reductions in the levels of NICD in primary AML samples, indicating that EGFL7 inhibits NOTCH activation (**Figure 2A, Figure S5A**). To test whether EGFL7-mediated down regulation of NOTCH activity results in decreased expression of NOTCH downstream target genes, we assessed one of the best characterized NOTCH target genes, *Hairy and Enhancer of Split-1* (*HES1)* (13,17). Primary AML patient samples stimulated with rEGFL7 for 24 hours results in decreased levels of *HES1* mRNA in primary AML blasts (n=3, **Figure 2B**).

Inhibition of EGFL7 results in NOTCH activation

Next, we examined whether NOTCH reactivation could be achieved by blocking EGFL7. We first measured activated NICD levels 4 hours after treatment with anti-EGFL7 and found significant increases in NICD levels in primary AML samples (n=3, **Figure 3A, Figure S5B**). We also found concomitant increases in the expression of *HES1* at 2 and 10h post anti-EGFL7 treatment (**Figure 3B**). In addition, we found increases in two other well-known NOTCH targets, *HEY1* and *DTX1* mRNA. (Supplemental Materials **Figure**

S6). Altogether these data support the role of EGFL7 in blocking NOTCH activation in primary AML blasts.

EGFL7 prevents NOTCH ligand induced NOTCH activation in AML cells

To determine whether EGFL7 inhibits NOTCH signaling in AML cells, we tested whether EGFL7 competes with canonical NOTCH ligands for receptor binding. To perform these experiments, we cultured THP-1 cells on DLL4-Fc and IgG-Fc (control) coated plates to activate NOTCH signaling and then tested the effect of rEGFL7 and anti-EGFL7 on this DLL4-Fc-mediated NOTCH activation in THP-1 cells (**Figure 4A**). We found that THP-1 cells cultured on DLL4-Fc-coated plates resulted in significant increases of NOTCH activation, assessed by *HES1* expression compared to cells cultured on IgG-Fc-coated control plates. Addition of γ-secretase inhibitor (AVA) significantly reduced DLL4-Fc-mediated NOTCH activation, while addition of an anti-EGFL7 blocking antibody enhanced DLL4-Fc-mediated NOTCH activation (**Figure 4B-C**). Similar experiments were performed in primary AML blasts (n=3). We found that DLL4-Fc pretreatment activated NOTCH and was enhanced by anti-EGFL7 antibody. Similar to the cell line, AVA treatment of primary AML patient blasts significantly prevented DLL4-Fc-mediated NOTCH activation. The treatment with rEGFL7 showed analogous results although the decrease was not as strong as chemical inhibition with AVA (**Figure 4D**). To validate these results, we used another well-known NOTCH ligand DLL1 (see also Supplemental Materials). For these experiments, we performed co-culture of THP-1 cells with stromal cells (OP9) overexpressing membrane bound DLL1 (OP9-DLL1) (26) (**Figure S7**). We found that similar to DLL4-FC, THP-1 cells co-cultured with OP9-DLL1 also results in significant increases in NOTCH activation, assessed by *HES1* expression (**Figure S7B,** OP9 vs OP9-DLL1, $P<0.001$). Addition of rEGFL7 or AVA significantly reduced DLL1-mediated NOTCH activation (**Figure S7B**, OP9-DLL1 vs OP9-DLL1 +rE7, $P<0.01$). In

addition, both rEGFL7 and AVA reduced NOTCH activation in THP-1 cells co-cultured with OP9 cells ($P<0.01$ and $P<0.05$, respectively).

Inhibition of EGFL7 results in increased blast cell apoptosis and differentiation

We have previously shown that anti-EGFL7 treatment of AML cells resulted in decreased blast growth and survival (5). To determine whether reactivation of NOTCH in AML cells leads to leukemic cell differentiation and survival, we treated THP-1 cells with anti-EGFL7 and measured apoptosis and differentiation. We found an increase in apoptotic cells after anti-EGFL7 treatment (**Figure 5A** and **Figure S8A**). These findings were supported by western blots demonstrating an increase in PARP1 and CASP3 cleavage as a result of anti-EGFL7 treatment (**Figure 5B**). To determine whether anti-EGFL7 also results in cell differentiation of THP-1 cells, we measured the levels of differentiation markers (CD11b and CD14) and found increases in both markers at 96-hours post anti-EGFL7 treatment (**Figure 5C and Figure S8B**). To determine whether blocking EGFL7 and subsequent activation by DLL4 results in enhanced cell death of AML cells, we cultured THP-1 cells with anti-EGFL7 followed by NOTCH activation using DLL4-Fc or IgG-Fc control. We found significant increases in apoptosis in THP-1 cells treated with anti-EGFL7 + DLL4-Fc compared to either DLL4-Fc or anti-EGFL7 alone (**Figure 5D**), suggesting that blocking EGFL7 may be a prerequisite for subsequent NOTCH activation in AML cells. In summary, these data demonstrate the ability of EGFL7 to block key NOTCH ligand binding and results in inhibition of canonical NOTCH signaling. Furthermore, EGFL7-mediated antagonism of NOTCH activation is reversible in AML, and can be re-activated by blocking EGFL7 with a monoclonal antibody (**Figure S9**).

Anti-EGFL7 antibody activates Notch signaling *in vivo* and prolongs survival in AML mouse models

Since THP-1 xenografts do not develop a lethal leukemia, and both THP-1 and EOL-1 cells have significant levels of EGFL7 expression (**Figure S1**),(5) we chose the EOL-1 xenograft AML model to test the feasibility of targeting EGFL7 *in vivo*. Briefly, $10X10^6$ EOL-1 cells were transplanted into non-conditioned NSG recipient mice. Two weeks post-transplantation, mice were treated with 50mg/kg body weight of anti-EGFL7 or IgG1 control antibody 3 times per week (**Figure 6A**). We found that treatment with anti-EGFL7 prolonged survival compared to IgG1 controls (*P*=0.01, **Figure 6B**). Furthermore, we found *in vivo* anti-EGFL7 antibody treatment resulted in reactivation of NOTCH signaling as demonstrated by increased levels of HES1 protein via western blots compared to IgG1 controls (**Figure 6C**). To further strengthen these findings, we used our well-established $Mll^{PTD/WT}$ $Flt3^{ITD/WT}$ double knock-in AML mouse model (**Figure 6D**)[5] . We found that treatment with the anti-EGFL7 antibody prolonged the life of the mice significantly compared to IgG1 control treated mice(*P*=0.002, **Figure 6E**). Finally, using a PDX model derived from cells of AML patient 5 (**Figure 6F**), we also observed prolonged survival of the anti-EGFL7 treated mice (*P*=0.04, **Figure 6G**). Overall, these results demonstrate that EGFL7 targeted therapy leads to NOTCH reactivation *in vivo* and represents a novel therapy for patients with AML.

DISCUSSION

EGFL7 is a protein that is secreted by endothelial cells to promote cell growth and migration (2,25,27-29). Several other tissues also express EGFL7 at low levels, but its expression can be induced during angiogenesis and in response to hypoxia, and vasculature injury (1,23,30). The Egfl7:eGFP transgenic mouse reporter strain demonstrated that *Egfl7* expression highly correlated with active sites of angiogenesis

(1). Recently, the role of *EGFL7* overexpression has been shown to be clinically important and correlates with tumor grade in many solid tumors such as: laryngeal squamous cell carcinoma, malignant glioma, breast cancer, and hepatocellular carcinoma (31,32). In many cases the mechanism of EGFL7-mediated tumorigenesis is believed to involve EGFL7's role to induce vessel formation through regulation of endothelial cell functions such as: proliferation, survival, and/or migration (2,25). Recently, we have described a role for EGFL7 in AML (5). We found that the levels of both EGFL7 mRNA and protein are increased in primary AML blasts compared to normal bone marrow cells. Moreover, high *EGFL7* mRNA expression associated with lower complete remission rates, shorter event-free and shorter overall survival in older (aged ≥60 years) and younger (aged <60 years) patients with cytogenetically normal AML. We further showed that AML blasts secrete EGFL7 protein, and that increased levels of EGFL7 protein are found in the sera from AML patients compared to sera from healthy controls. Treatment of primary AML blasts with recombinant rEGFL7 *in vitro* leads to increases in leukemic blast cell growth (5). However, the molecular mechanisms underlying EGFL7 induced leukemogenesis has not been fully elucidated. Here, we demonstrate for the first time that EGFL7 is able to directly bind multiple proteins on primary AML blasts, including NOTCH. EGFL7 has previously been shown to bind NOTCH receptors and can both activate and repress NOTCH activation depending on the tissue cell type. Whether EGFL7 activates or represses NOTCH activity in AML has not previously been described. Our data now suggests that EGFL7 contributes to leukemogenesis, at least in part, through antagonizing NOTCH signaling (2,25).

NOTCH signaling is important for normal hematopoiesis, and oncogenic activation of NOTCH signaling has been shown to contribute to leukemogenesis in T-ALL (33-37). However, there is still uncertainty when it comes to the exact role of NOTCH in different

hematopoietic compartments. Previously, other groups have shown that the NOTCH signaling pathway is silenced in AML (17,18). Lobry *et al.* demonstrated that NOTCH re-activation by DLL4 induced the expression of apoptosis- and differentiation-associated genes, including *HES1*, *BCL2*, *ADAMDEC1*, *CD11C*, *CD74*, and *CDKN1A* (17). Consequently, they found that NOTCH re-activation in AML blasts led to increased differentiation and apoptosis. However, the mechanism underlying the NOTCH inactivation in AML has remained to be further explained (16-18). It is possible that decreased NOTCH activation could be the result of decreased levels of NOTCH ligands, insufficient levels of NOTCH receptor expression, or inadequate cleavage and/or translocation of NICD to the nucleus to activate target gene transcription. Here, we propose one possible mechanism by which the NOTCH pathway remains inactive in AML is through EGFL7 antagonism of normal NOTCH ligand binding to NOTCH receptors on leukemic blasts (**Figure S9**). However, antagonism of NOTCH by EGFL7 binding might not be the only mechanism involving EGFL7-mediated silencing of NOTCH signaling. We found that in response to EGFL7 stimulation, primary AML blasts had decreased cleavage of NOTCH at 4 hours post stimulation. Interestingly, at 24 hours post stimulation, we found significant decreases in the levels of *NOTCH* mRNA. These results suggest that EGFL7 binding to NOTCH receptors may also lead to long-term transcriptional repression of *NOTCH*, creating a negative feedback loop involving EGFL7, NOTCH signaling inactivation, and *NOTCH* transcriptional repression. The exact mechanism by which EGFL7 stimulation leads to transcriptional repression of *NOTCH* mRNA is currently unknown, but future studies to determine how this is regulated could provide novel insights into NOTCH gene regulation.

Although the interaction of EGFL7 with a critical signaling pathway such as NOTCH is important for understanding the cross regulation of these pathways in AML, the results

from our antibody array demonstrate the ability of EGFL7 to bind many proteins important for regulating diverse biological processes. Therefore, it seems likely that EGFL7 might be functioning in many different signaling pathways important for AML. Future studies examining how these pathways may be working in synergy in AML blasts will be important to determine the extent by which EGFL7 influences AML biology and the importance of developing anti-EGFL7 therapies to treat patients with AML.

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AUTHORSHIP CONTRIBUTIONS

M.B. and A.M.D. designed the study. M.B., A.P., M.K., C.S., M.H.B., P.R., D.P., N.Z., K.S., A.LR., A.E.W., Z.J.B., A.P.N., A.G.F., M.D., X.Z., C.D.B., R.G., and A.M.D performed experiments and contributed to data interpretation. M.B., A.P., M.K., R.G., and A.M.D. wrote the manuscript. All authors reviewed the manuscript and approved the final version.

REFERENCES

1. Bambino K, Lacko LA, Hajjar KA, Stuhlmann H. Epidermal growth factor-like domain 7 is a marker of the endothelial lineage and active angiogenesis. Genesis **2014**;52(7):657-70 doi 10.1002/dvg.22781.
2. Nichol D, Stuhlmann H. EGFL7: a unique angiogenic signaling factor in vascular development and disease. Blood **2012**;119(6):1345-52 doi 10.1182/blood-2011-10-322446.
3. Oh J, Park SH, Lee TS, Oh HK, Choi JH, Choi YS. High expression of epidermal growth factor-like domain 7 is correlated with poor differentiation and poor prognosis in patients with epithelial ovarian cancer. J Gynecol Oncol **2014**;25(4):334-41 doi 10.3802/jgo.2014.25.4.334.
4. Fan C, Yang LY, Wu F, Tao YM, Liu LS, Zhang JF*, et al.* The expression of Egfl7 in human normal tissues and epithelial tumors. Int J Biol Markers **2013**;28(1):71-83 doi 10.5301/JBM.2013.10568.
5. Papaioannou D, Shen C, Nicolet D, McNeil B, Bill M, Karunasiri M*, et al.* Prognostic and biological significance of the proangiogenic factor EGFL7 in acute myeloid leukemia. Proc Natl Acad Sci U S A **2017**;114(23):E4641-E7 doi 10.1073/pnas.1703142114.
6. Schmidt MH, Bicker F, Nikolic I, Meister J, Babuke T, Picuric S*, et al.* Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. Nat Cell Biol **2009**;11(7):873-80 doi 10.1038/ncb1896.
7. Massimiani M, Vecchione L, Piccirilli D, Spitalieri P, Amati F, Salvi S*, et al.* Epidermal growth factor-like domain 7 promotes migration and invasion of human trophoblast cells through activation of MAPK, PI3K and NOTCH signaling pathways. Mol Hum Reprod **2015**;21(5):435-51 doi 10.1093/molehr/gav006.
8. Heidel FH, Arreba-Tutusaus P, Armstrong SA, Fischer T. Evolutionarily conserved signaling pathways: acting in the shadows of acute myelogenous leukemia's genetic diversity. Clin Cancer Res **2015**;21(2):240-8 doi 10.1158/1078-0432.CCR-14-1436.
9. Briot A, Bouloumie A, Iruela-Arispe ML. Notch, lipids, and endothelial cells. Curr Opin Lipidol **2016** doi 10.1097/MOL.0000000000000337.
10. Acar A, Simoes BM, Clarke RB, Brennan K. A Role for Notch Signalling in Breast Cancer and Endocrine Resistance. Stem Cells Int **2016**;2016:2498764 doi 10.1155/2016/2498764.

11. Lu J, Xia Y, Chen K, Zheng Y, Wang J, Lu W*, et al.* Oncogenic role of the Notch pathway in primary liver cancer. Oncol Lett **2016**;12(1):3-10 doi 10.3892/ol.2016.4609.
12. D'Souza B, Meloty-Kapella L, Weinmaster G. Canonical and non-canonical Notch ligands. Curr Top Dev Biol **2010**;92:73-129 doi 10.1016/S0070-2153(10)92003-6.
13. Lobry C, Oh P, Mansour MR, Look AT, Aifantis I. Notch signaling: switching an oncogene to a tumor suppressor. Blood **2014**;123(16):2451-9 doi 10.1182/blood-2013-08-355818.
14. Ntziachristos P, Lim JS, Sage J, Aifantis I. From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell **2014**;25(3):318-34 doi 10.1016/j.ccr.2014.02.018.
15. Oh P, Lobry C, Gao J, Tikhonova A, Loizou E, Manet J*, et al.* In vivo mapping of notch pathway activity in normal and stress hematopoiesis. Cell Stem Cell **2013**;13(2):190-204 doi 10.1016/j.stem.2013.05.015.
16. Kannan S, Sutphin RM, Hall MG, Golfman LS, Fang W, Nolo RM*, et al.* Notch activation inhibits AML growth and survival: a potential therapeutic approach. J Exp Med **2013**;210(2):321-37 doi 10.1084/jem.20121527.
17. Lobry C, Ntziachristos P, Ndiaye-Lobry D, Oh P, Cimmino L, Zhu N*, et al.* Notch pathway activation targets AML-initiating cell homeostasis and differentiation. J Exp Med **2013**;210(2):301-19 doi 10.1084/jem.20121484.
18. Kato T, Sakata-Yanagimoto M, Nishikii H, Ueno M, Miyake Y, Yokoyama Y*, et al.* Hes1 suppresses acute myeloid leukemia development through FLT3 repression. Leukemia **2015**;29(3):576-85 doi 10.1038/leu.2014.281.
19. von Pawel J, Spigel DR, Ervin T, Losonczy G, Barlesi F, Juhasz E*, et al.* Randomized Phase II Trial of Parsatuzumab (Anti-EGFL7) or Placebo in Combination with Carboplatin, Paclitaxel, and Bevacizumab for First-Line Nonsquamous Non-Small Cell Lung Cancer. Oncologist **2018**;23(6):654-e58 doi 10.1634/theoncologist.2017-0690.
20. Dorrance AM, De Vita S, Radu M, Reddy PN, McGuinness MK, Harris CE*, et al.* The Rac GTPase effector p21-activated kinase is essential for hematopoietic stem/progenitor cell migration and engraftment. Blood **2013**;121(13):2474-82 doi 10.1182/blood-2012-10-460709.

21. Nikolic I, Stankovic ND, Bicker F, Meister J, Braun H, Awwad K*, et al.* EGFL7 ligates alphavbeta3 integrin to enhance vessel formation. Blood **2013**;121(15):3041-50 doi 10.1182/blood-2011-11-394882.
22. Renz M, Otten C, Faurobert E, Rudolph F, Zhu Y, Boulday G*, et al.* Regulation of beta1 integrin-Klf2-mediated angiogenesis by CCM proteins. Dev Cell **2015**;32(2):181-90 doi 10.1016/j.devcel.2014.12.016.
23. Badiwala MV, Tumiati LC, Joseph JM, Sheshgiri R, Ross HJ, Delgado DH*, et al.* Epidermal growth factor-like domain 7 suppresses intercellular adhesion molecule 1 expression in response to hypoxia/reoxygenation injury in human coronary artery endothelial cells. Circulation **2010**;122(11 Suppl):S156-61 doi 10.1161/CIRCULATIONAHA.109.927715.
24. Badiwala MV, Guha D, Tumiati L, Joseph J, Ghashghai A, Ross HJ*, et al.* Epidermal growth factor-like domain 7 is a novel inhibitor of neutrophil adhesion to coronary artery endothelial cells injured by calcineurin inhibition. Circulation **2011**;124(11 Suppl):S197-203 doi 10.1161/CIRCULATIONAHA.110.011734.
25. Nichol D, Shawber C, Fitch MJ, Bambino K, Sharma A, Kitajewski J*, et al.* Impaired angiogenesis and altered Notch signaling in mice overexpressing endothelial Egfl7. Blood **2010**;116(26):6133-43 doi 10.1182/blood-2010-03-274860.
26. Schmitt TM, de Pooter RF, Gronski MA, Cho SK, Ohashi PS, Zuniga-Pflucker JC. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol **2004**;5(4):410-7 doi 10.1038/ni1055.
27. Luo BH, Xiong F, Wang JP, Li JH, Zhong M, Liu QL*, et al.* Epidermal growth factor-like domain-containing protein 7 (EGFL7) enhances EGF receptor-AKT signaling, epithelial-mesenchymal transition, and metastasis of gastric cancer cells. PLoS One **2014**;9(6):e99922 doi 10.1371/journal.pone.0099922.
28. Shen X, Han Y, Xue X, Li W, Guo X, Li P*, et al.* Epidermal growth factor-like domain 7 promotes cell invasion and angiogenesis in pancreatic carcinoma. Biomed Pharmacother **2016**;77:167-75 doi 10.1016/j.biopha.2015.12.009.
29. Wang XX, Yao XB, Qiang ZS, Zhu HL. Attenuation of EGFL7 inhibits human laryngocarcinoma cells growth and invasion. Int J Clin Exp Med **2015**;8(3):3141-55.

30. Johnson L, Huseni M, Smyczek T, Lima A, Yeung S, Cheng JH*, et al.* Anti-EGFL7 antibodies enhance stress-induced endothelial cell death and anti-VEGF efficacy. J Clin Invest **2013**;123(9):3997-4009 doi 10.1172/JCI67892.
31. Li JJ, Yang XM, Wang SH, Tang QL. Prognostic role of epidermal growth factor-like domain 7 protein expression in laryngeal squamous cell carcinoma. J Laryngol Otol **2011**;125(11):1152-7 doi 10.1017/S0022215111002441.
32. Huang CH, Li XJ, Zhou YZ, Luo Y, Li C, Yuan XR. Expression and clinical significance of EGFL7 in malignant glioma. J Cancer Res Clin Oncol **2010**;136(11):1737-43 doi 10.1007/s00432-010-0832-9.
33. Hannon MM, Lohan F, Erbilgin Y, Sayitoglu M, O'Hagan K, Mills K*, et al.* Elevated TRIB2 with NOTCH1 activation in paediatric/adult T-ALL. Br J Haematol **2012**;158(5):626-34 doi 10.1111/j.1365-2141.2012.09222.x.
34. Wang W, Zimmerman G, Huang X, Yu S, Myers J, Wang Y*, et al.* Aberrant Notch Signaling in the Bone Marrow Microenvironment of Acute Lymphoid Leukemia Suppresses Osteoblast-Mediated Support of Hematopoietic Niche Function. Cancer Res **2016**;76(6):1641-52 doi 10.1158/0008-5472.CAN-15-2092.
35. Stein SJ, Mack EA, Rome KS, Pajcini KV, Ohtani T, Xu L*, et al.* Trib2 Suppresses Tumor Initiation in Notch-Driven T-ALL. PLoS One **2016**;11(5):e0155408 doi 10.1371/journal.pone.0155408.
36. Suresh S, Irvine AE. The NOTCH signaling pathway in normal and malignant blood cell production. J Cell Commun Signal **2015**;9(1):5-13 doi 10.1007/s12079-015-0271-0.
37. He Q, Zhang C, Wang L, Zhang P, Ma D, Lv J*, et al.* Inflammatory signaling regulates hematopoietic stem and progenitor cell emergence in vertebrates. Blood **2015**;125(7):1098-106 doi 10.1182/blood-2014-09-601542.

FIGURE LEGENDS

Figure 1. A Cell lysate of $5x10^6$ primary AML blasts (ptAML; n=3) was incubated with an antibody array containing antibodies against 400 signal molecules, followed by sequential incubation with 10 nM human rEGFL7, HRP-conjugated anti-EGFL7 antibody (@EGFL7) or HRP-conjugated anti-GST antibody (control). The arrows show the identified NOTCH1 in the array membranes detected by HRP-conjugated anti-EGFL7 antibody but not HRP-conjugated anti-GST antibody. **B** Cell lysate of $10x10^6$ THP-1 cells or $5x10^6$ primary AML blasts were subjected to Co-IP with Protein G Plus/Protein A agarose beads. The eluate was analyzed by immunoblotting of EGFL7, NOTCH1 and NOTCH2 with the corresponding antibodies. **C** Cell lysate of $10x10^6$ K562 cells that were transfected with a MYC-tagged NOTCH2 and FLAG-tagged EGFL7 expression vectors, were subjected to Co-IP with Protein G Plus/Protein A agarose beads. The eluate was analyzed by immunoblotting of EGFL7 and NOTCH2 with the corresponding antibodies.

Figure 2. A Primary blasts (ptAML; n=3) were cultured in serum-free expansion medium (SFEM) with 20% fetal bovine serum (FBS) and cytokines in the presence or absence (unstimulated control; Unstim) of 0.25 μM rEGFL7 (+rEGFL7) for 4 h. Total proteins were extracted for immunoblotting using an NICD-specific antibody with β-ACTIN as loading control. **B** Total RNA was extracted for quantitative real time (qRT-PCR) analysis of *HES1* mRNA normalized to *β-ACTIN* control (n=3 individual AML patients after 24 hours of treatment. *P<0.05, **P<0.01, ***P<0.001) stimulated with +rEGFL7 vs Unstim.

Figure 3. A Primary blasts (ptAML; n=3) were cultured in presence of 100 μg/ml of normal IgG or 100 μg/ml anti-EGFL7 antibody (@EGFL7) for 4 h. Total protein was extracted for immunoblotting of NICD with β-ACTIN as loading control. **B** Primary blasts (n=3) were cultured in SFEM medium with 100 μg/ml of normal IgG or anti-EGFL7

antibody for 2 hours and 10 h. Total RNA was extracted for qRT-PCR analysis of *HES1* mRNA with *β-ACTIN* as internal control. **P*<0.05, ****P*<0.001 vs IgG control.

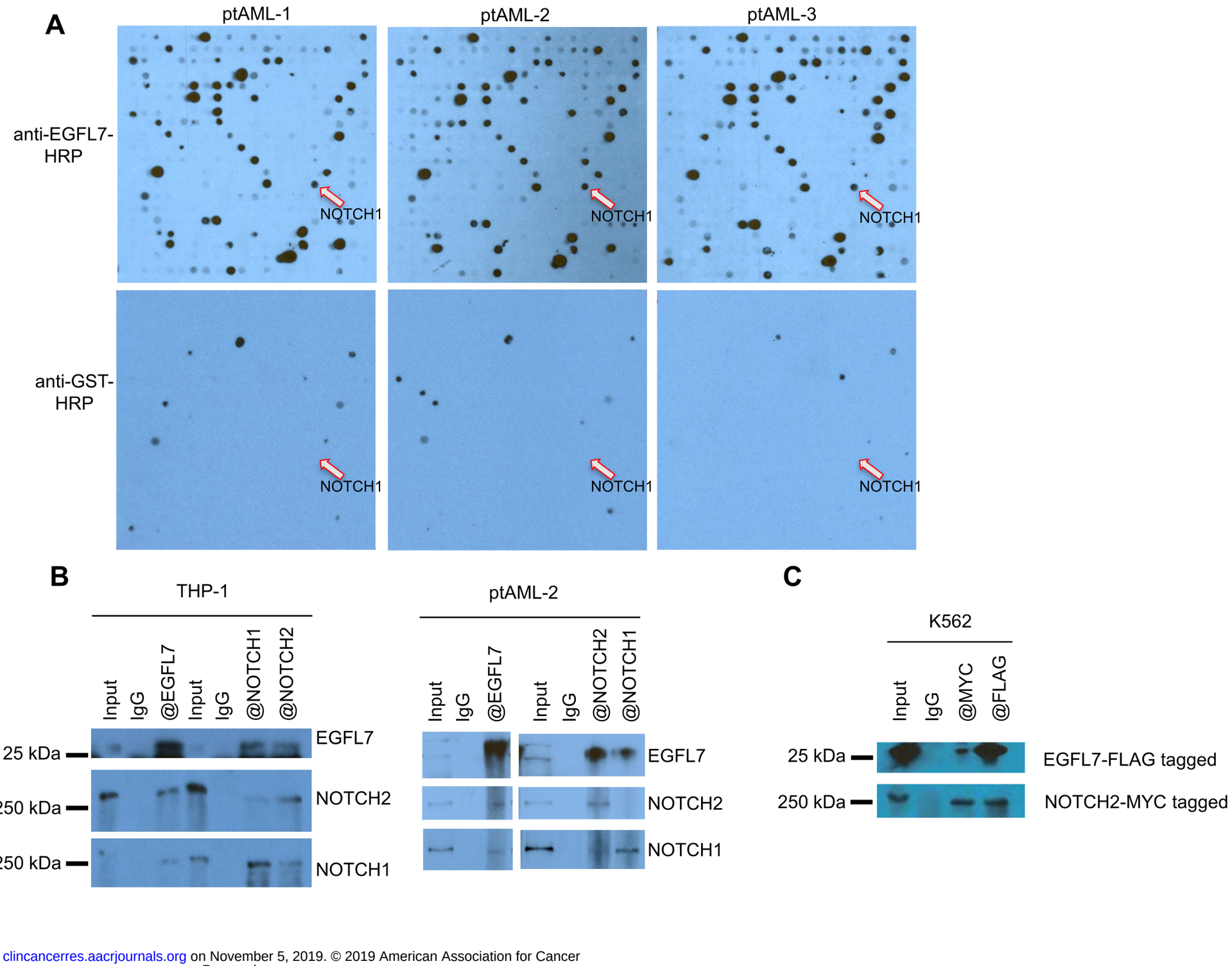
Figure 4. A Scheme of experiments using DELTA like (DLL) 4 Fc or IgG Fc control coated plates to induce NOTCH activation. **B** THP-1 cells were pre-treated with 10 μM γ-secretase inhibitor (Avagacestat [AVA]), 250 μg/ml anti-EGFL7 antibody (@EGFL7) or 1.0 μM rEGFL7 for 30 min, then transferred to IgG Fc or DLL4 Fc coated plates. After 4 hours of incubation total RNA from THP-1 cells was extracted for qRT-PCR analysis of *HES1* with *β-ACTIN* as internal control. $^{\#\#\#}$*P*<0.001 IgG Fc vs DLL4 Fc; ****P*<0.001 DLL4+AVA vs DLL4 Fc, DLL4+rEGFL7 vs DLL4 Fc, and DLL4+@EGFL7 vs DLL4 Fc. **C** Total proteins of similar treated cells were extracted for immunoblotting of NICD with β-ACTIN as loading control. **D** Primary AML blasts were pretreated with 10 μM AVA, 100 μg/ml anti-EGFL7 antibody or 0.25 μM rEGFL7 for 30 min, then transferred to IgG Fc or DLL4 Fc coated plates. After 4 hours of incubation, total RNA was extracted for RT-PCR analysis of *HES1* mRNA with *β-ACTIN* as internal control. $^{\#\#\#}$*P*<0.001 IgG Fc vs DLL4 Fc; **P*<0.05, ****P*<0.001 DLL4+AVA vs DLL4 Fc, DLL4+rEGFL7 vs DLL4 Fc, and DLL4+anti-EGFL7 vs DLL4 Fc.

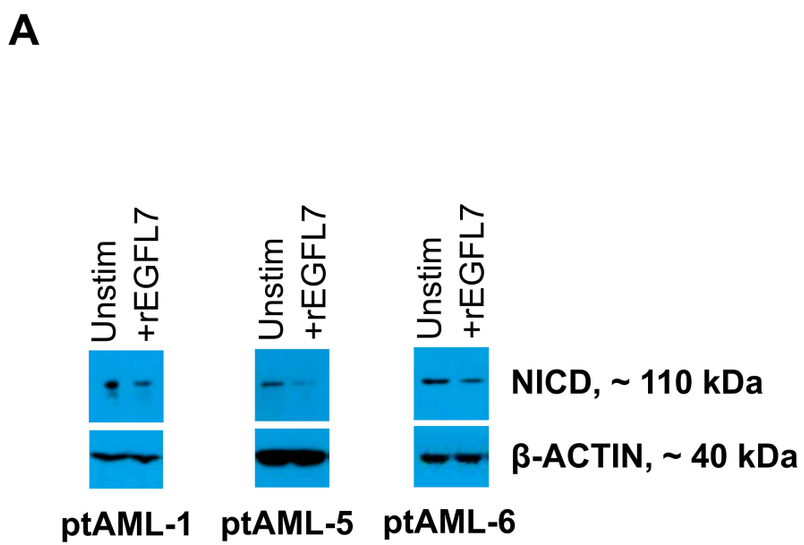
Figure 5. A THP-1 cells were treated at time 0 hours with the indicated concentration of anti-EGFL7 (@EGFL7). Cells were then assessed for apoptosis using AnnexinV+ staining and flow cytometry at 48 hours post treatment; **P*<0.05, ***P*<0.01 **B** Immunoblotting for full-length PARP1 (~115 kDa) and cleaved PARP1 (~90 kDa); full-length CASP3 (~30 kDa) and cleaved CASP3 (~17 and 19 kDa); GAPDH as loading control. **C** Cell differentiation was assessed by CD11B and CD14 staining 96-hours post anti-EGFL7 treatment; **P*<0.05; NS = not significant. **D** To determine whether activation

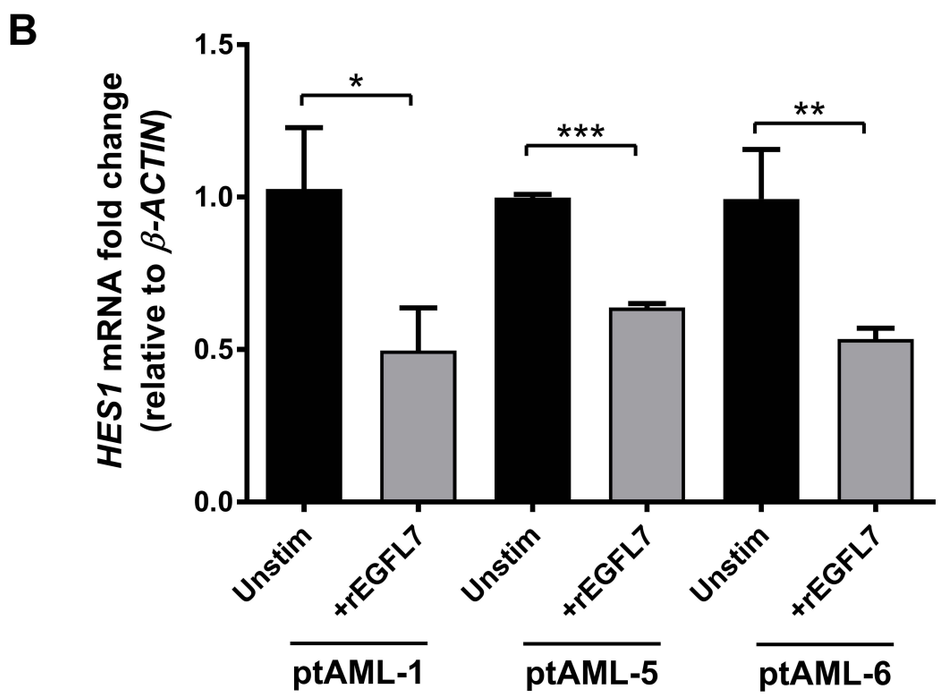
of NOTCH by DLL4 Fc could be accentuated by anti-EGFL7 treatment, we first treated THP-1 cells with anti-EGFL7 (100 µg/ml) and then cultured on DLL4 Fc or IgG Fc coated plates and then assessed for apoptosis using AnnexinV+ staining and flow cytometry. *P<0.05, **P<0.01, DLL4 Fc and anti-EGFL7; vs DLL4 Fc +anti-EGFL7; NS = not significant.

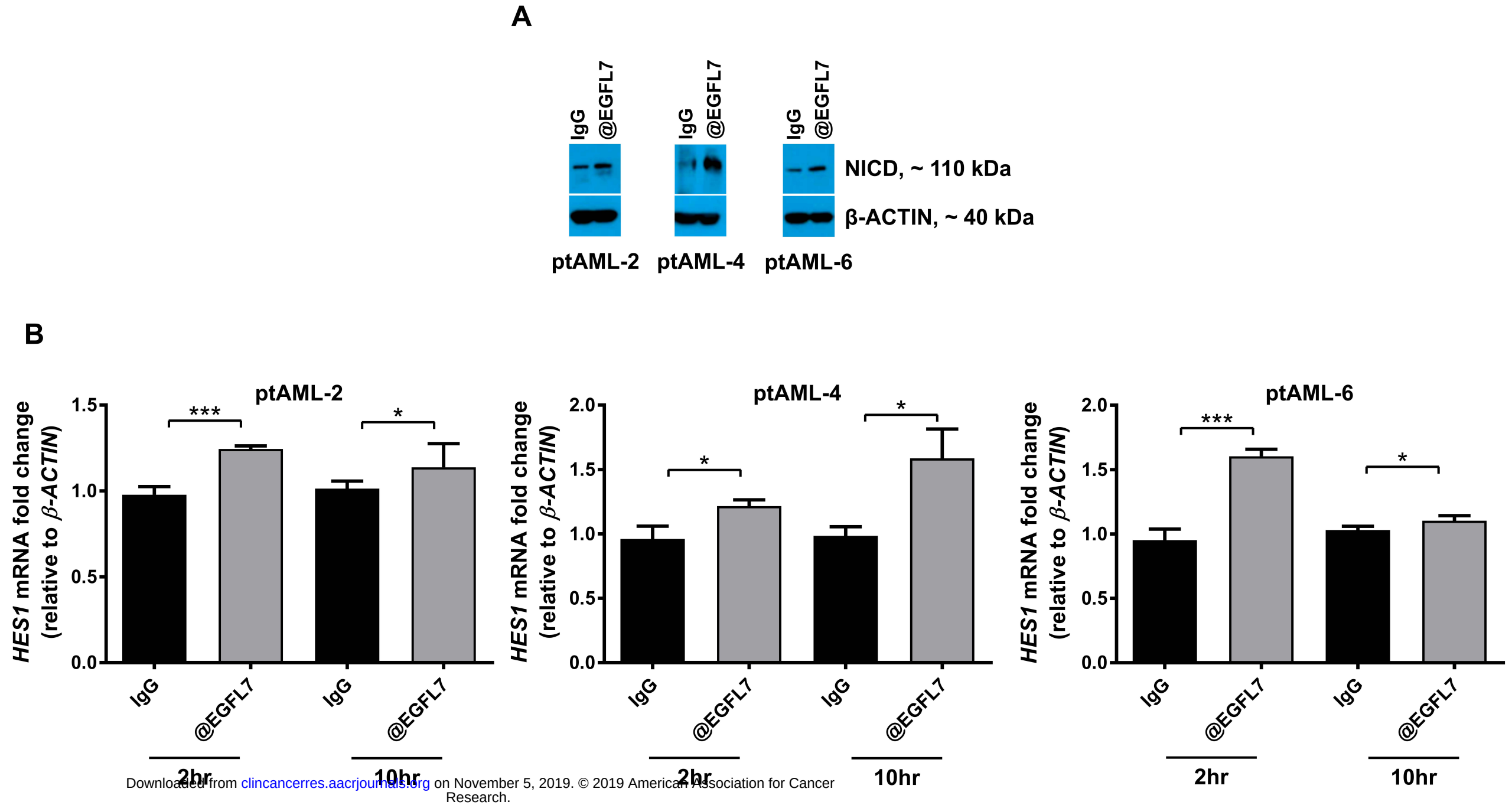
Figure 6. A Schematic representation of the experimental design using an EOL-1 xenograft AML mouse model. Briefly, $10X10^6$ EOL-1 cells were transplanted into non-conditioned NSG recipient mice (n=4 for each group). Two weeks post-transplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody 3 times per week. **B** Treatment with anti-EGFL7 blocking antibody prolonged survival compared to IgG1 controls (P=0.01). **C** *In vivo* treatment using an anti-EGFL7 antibody treatment results in increased levels of HES1 protein via western blots compared to IgG1 controls. **D** Schematic representation of the experimental design using our *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ double knock-in mouse model. $1X10^6$ primary AML bone marrow cells (CD45.2) together with $5X10^5$ healthy wild-type bone marrow cells (CD45.1) were transplanted into lethally irradiated BoyJ (CD45.1) recipient mice (n=7 for each group). Two weeks post-transplantation, mice were treated with 50 mg/kg body weight of anti-EGFL7 or IgG1 control antibody 2 times per week. **E** Anti-EGFL7 treatment prolonged survival of *Mll*$^{PTD/WT}$ *Flt3*$^{ITD/WT}$ transplanted mice compared to IgG1 controls (P=0.002). **F** Schematic representation of the experimental design using a patient derived xenotransplant (PDX) mouse model. Briefly, $2X10^6$ primary cells from a cytogenetically normal AML patient were transplanted into Busulfan-treated recipient mice (n=4 for each group). Four weeks post-transplantation, mice were treated with 50 mg/kg body weight

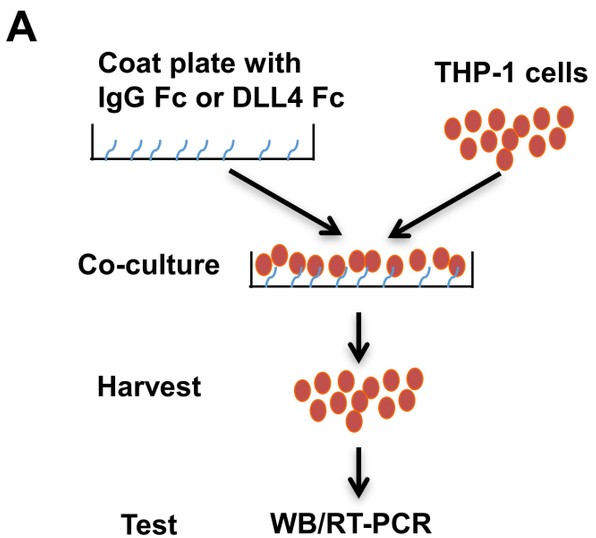
of anti-EGFL7 or IgG1 control antibody 2 times per week for 12 weeks. **G** We found that treatment with anti-EGFL7 prolonged survival compared to IgG1 controls (*P*=0.04).

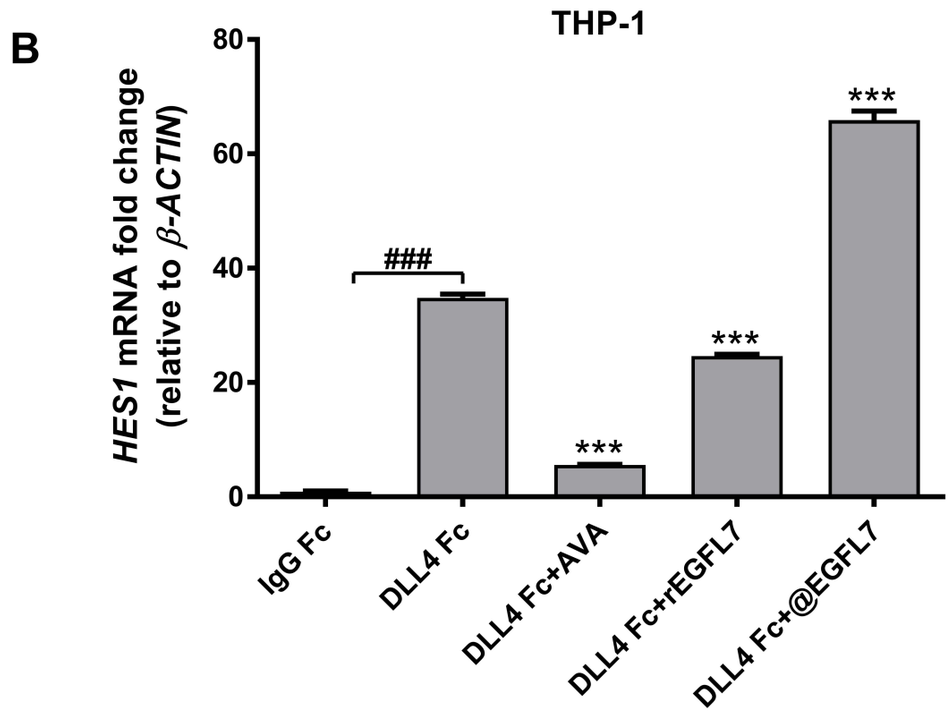


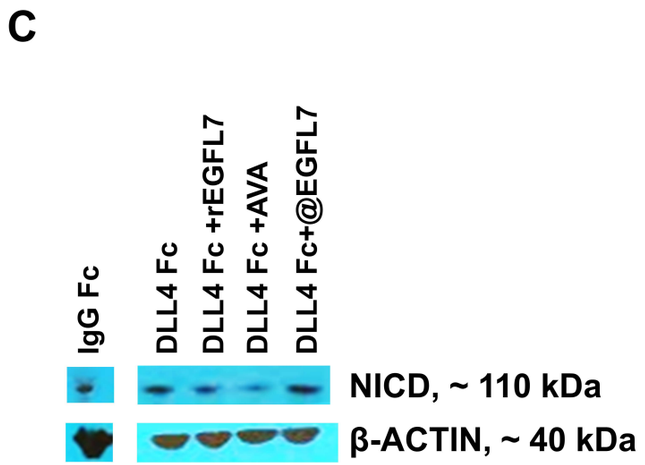


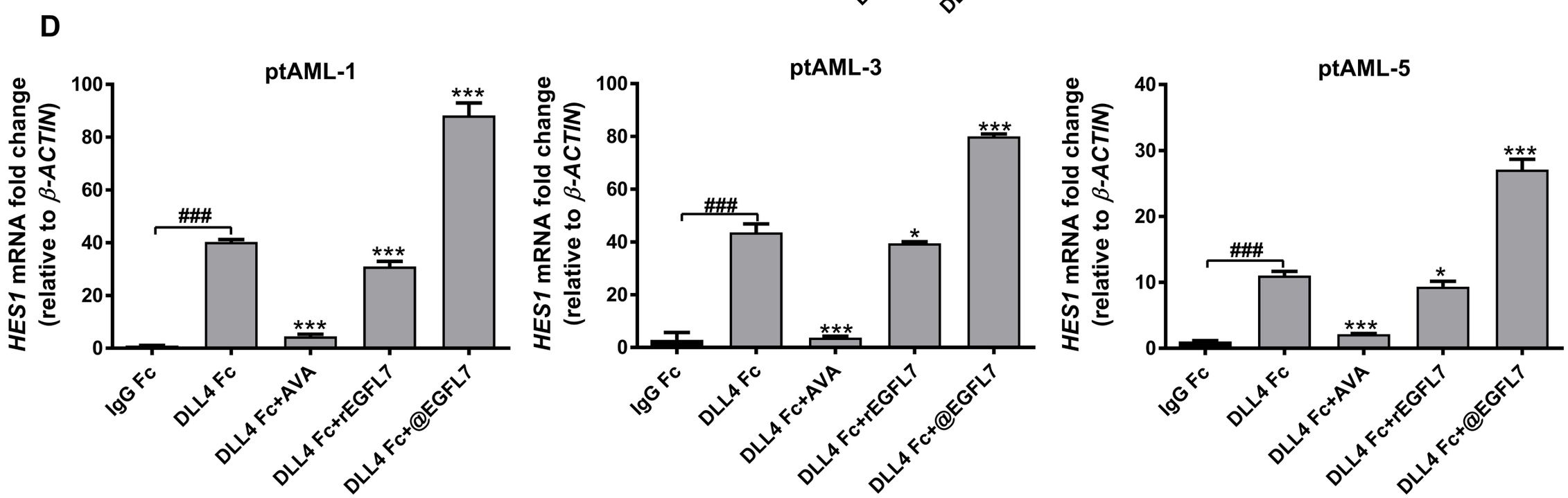


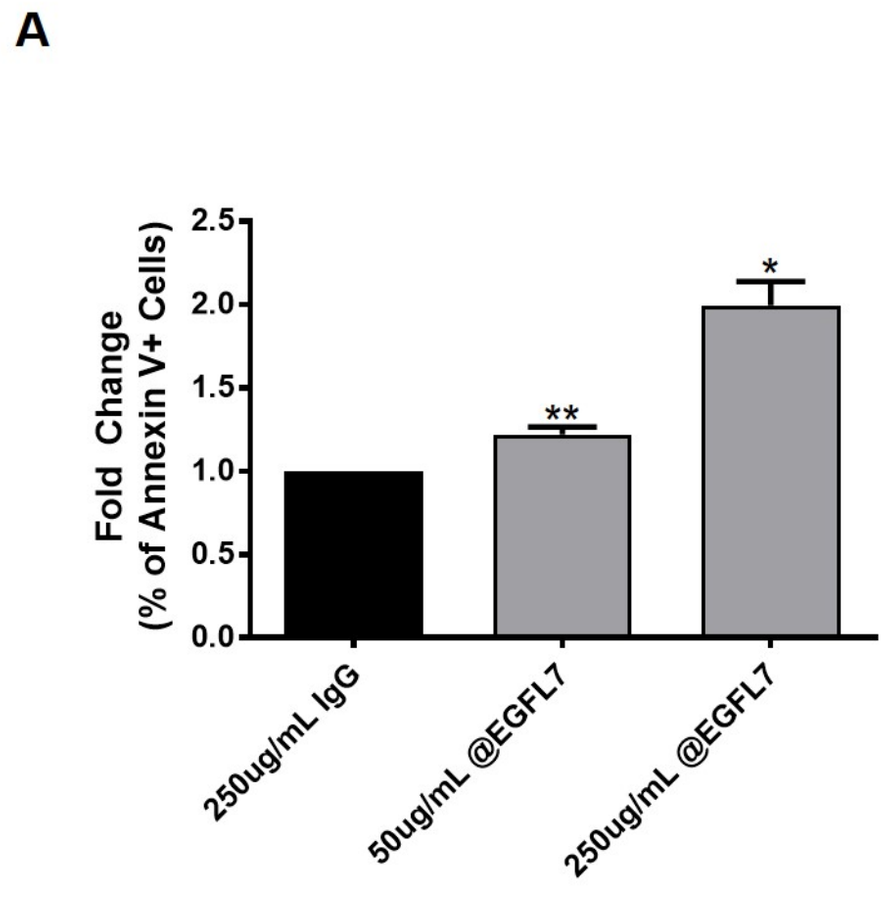


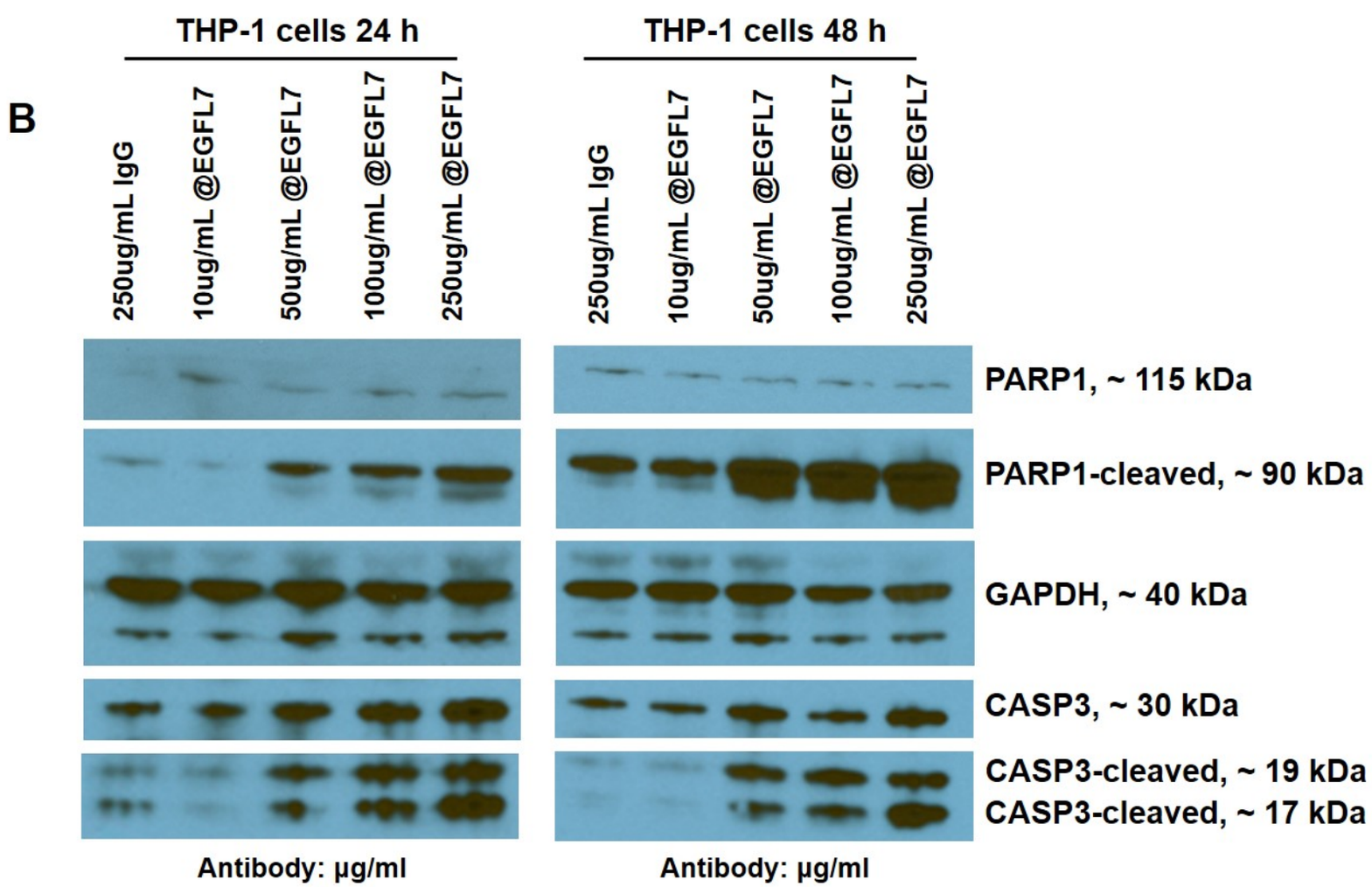


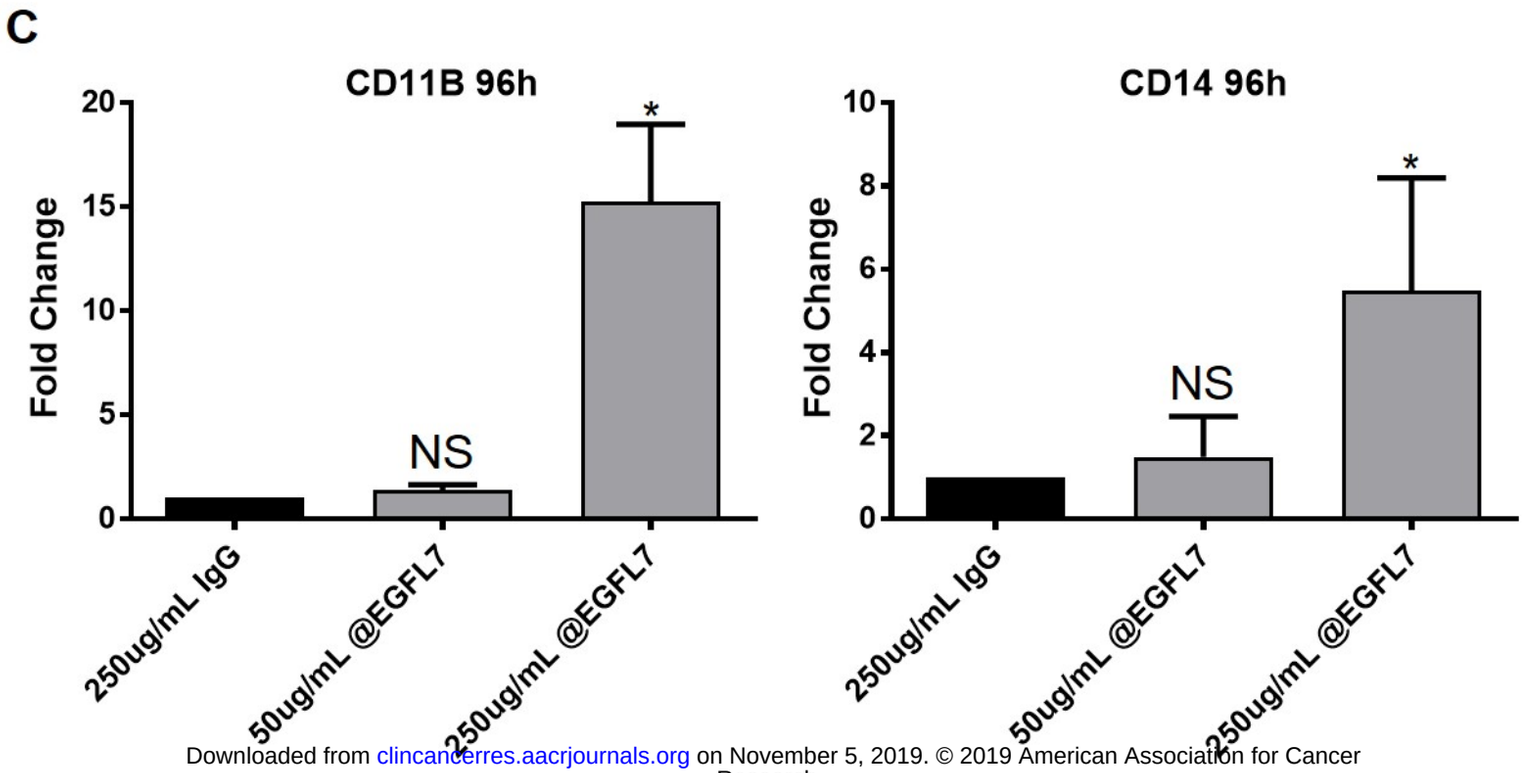


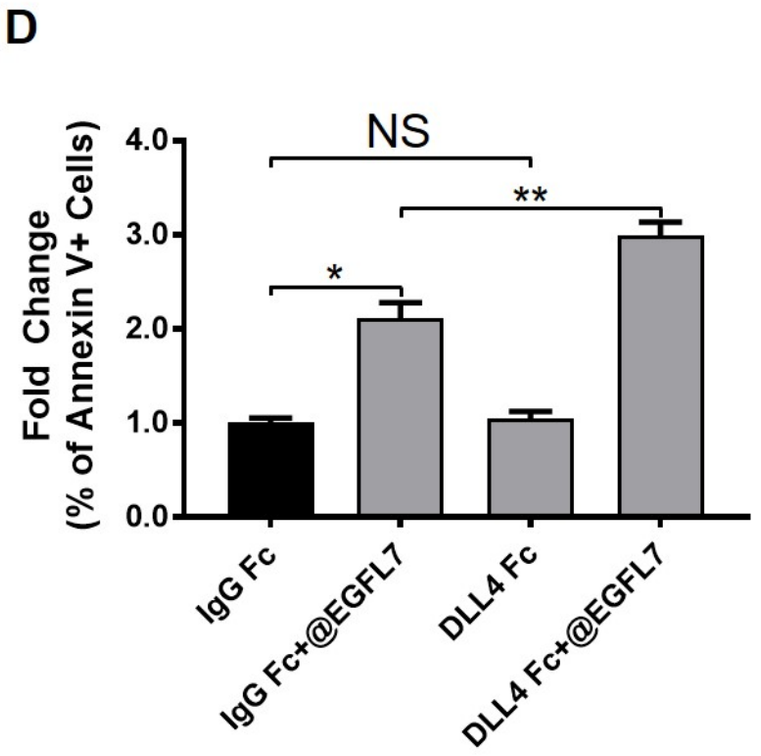


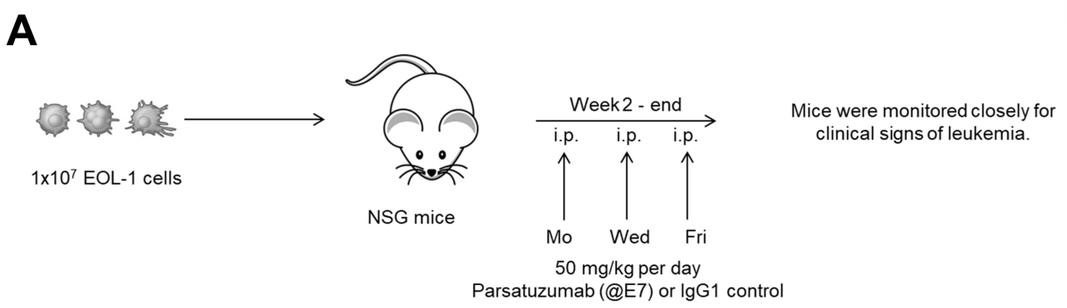


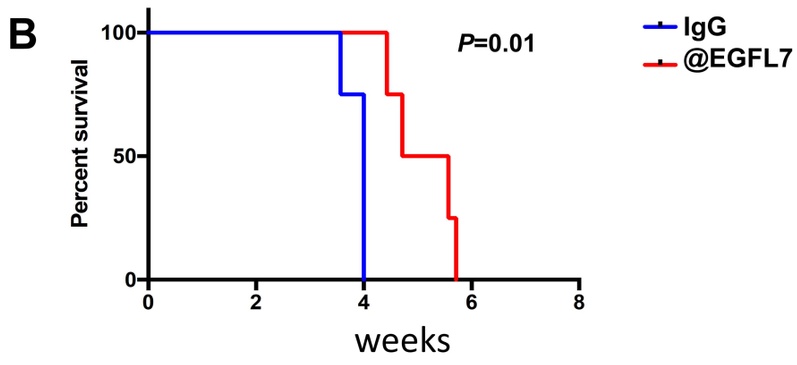


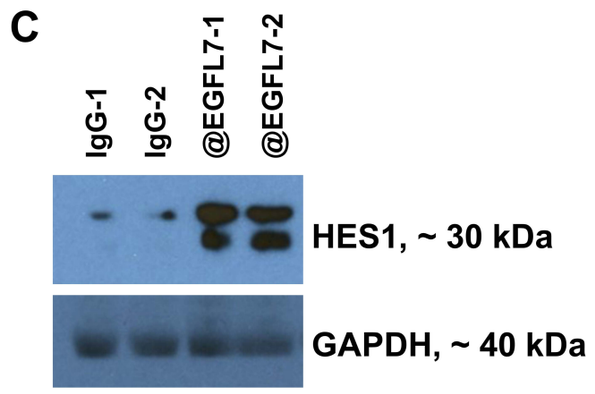


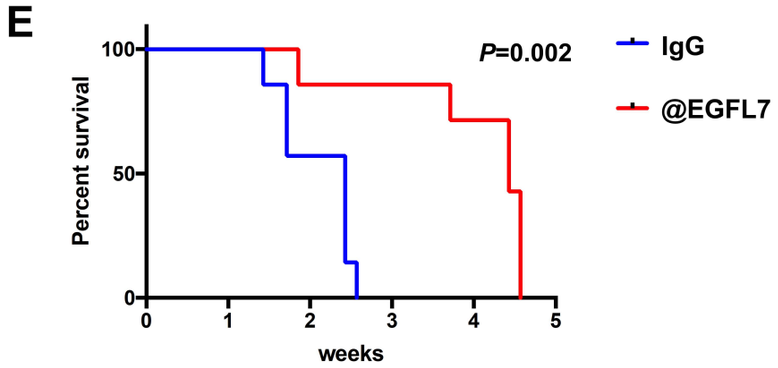


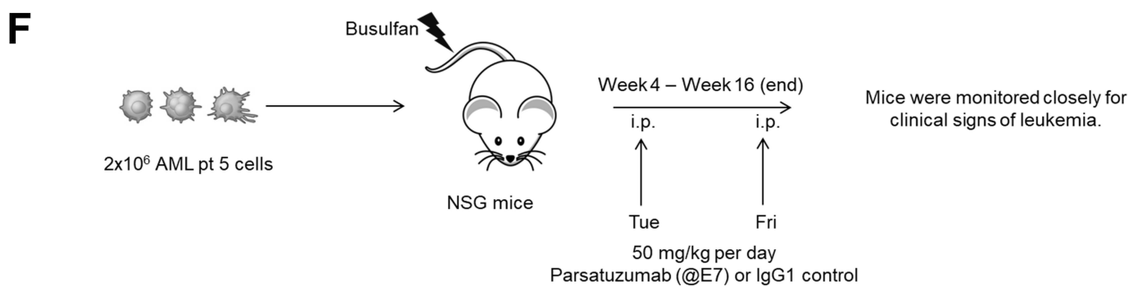


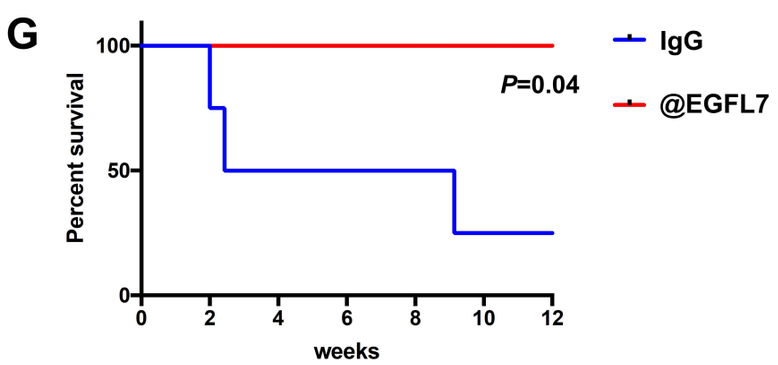












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