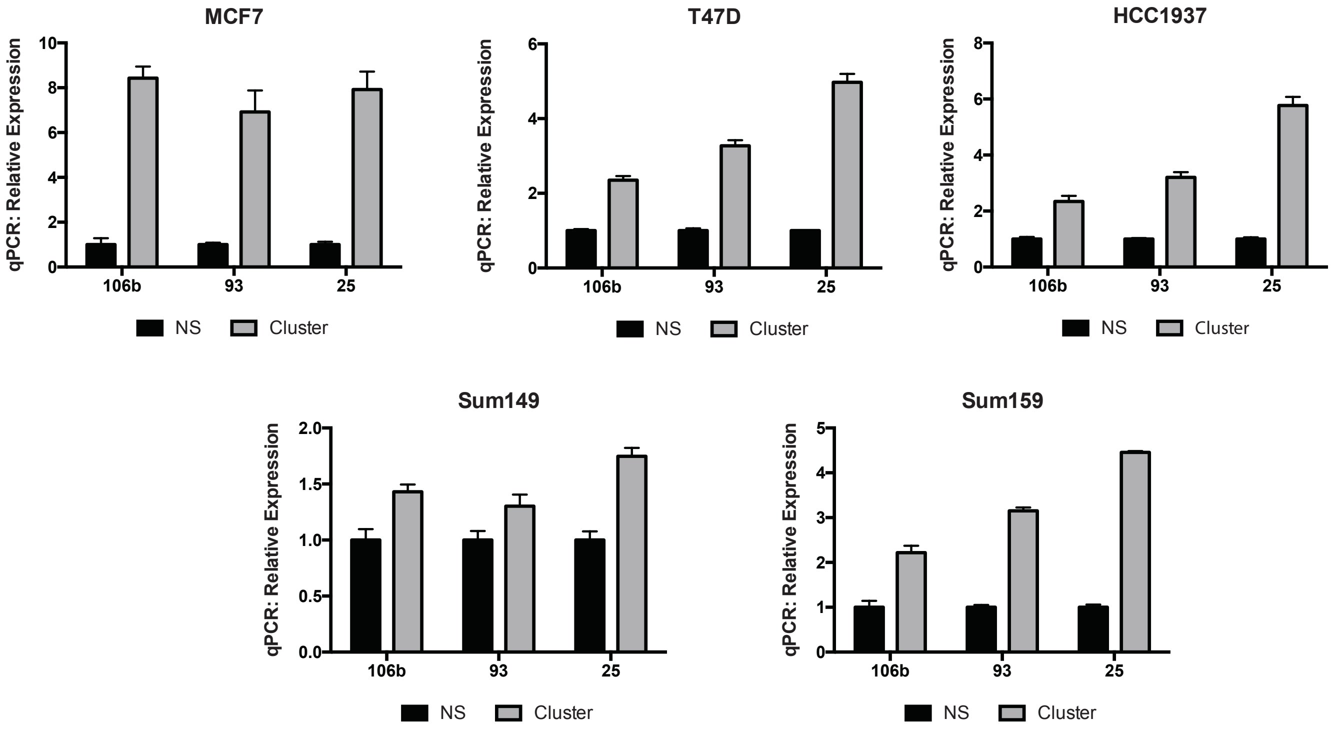
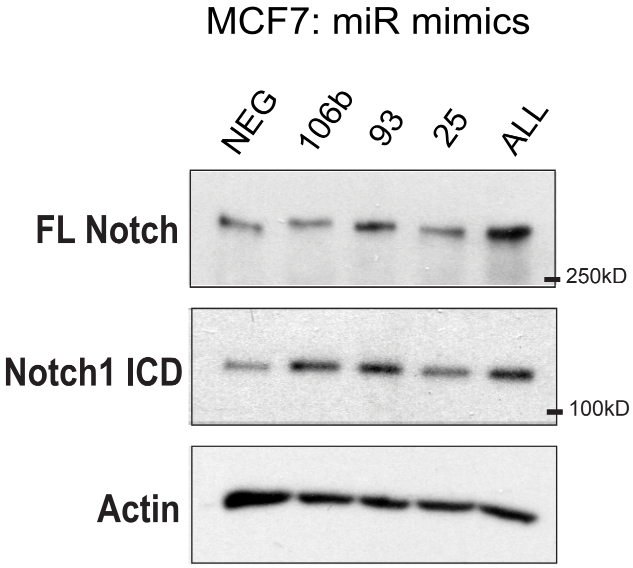
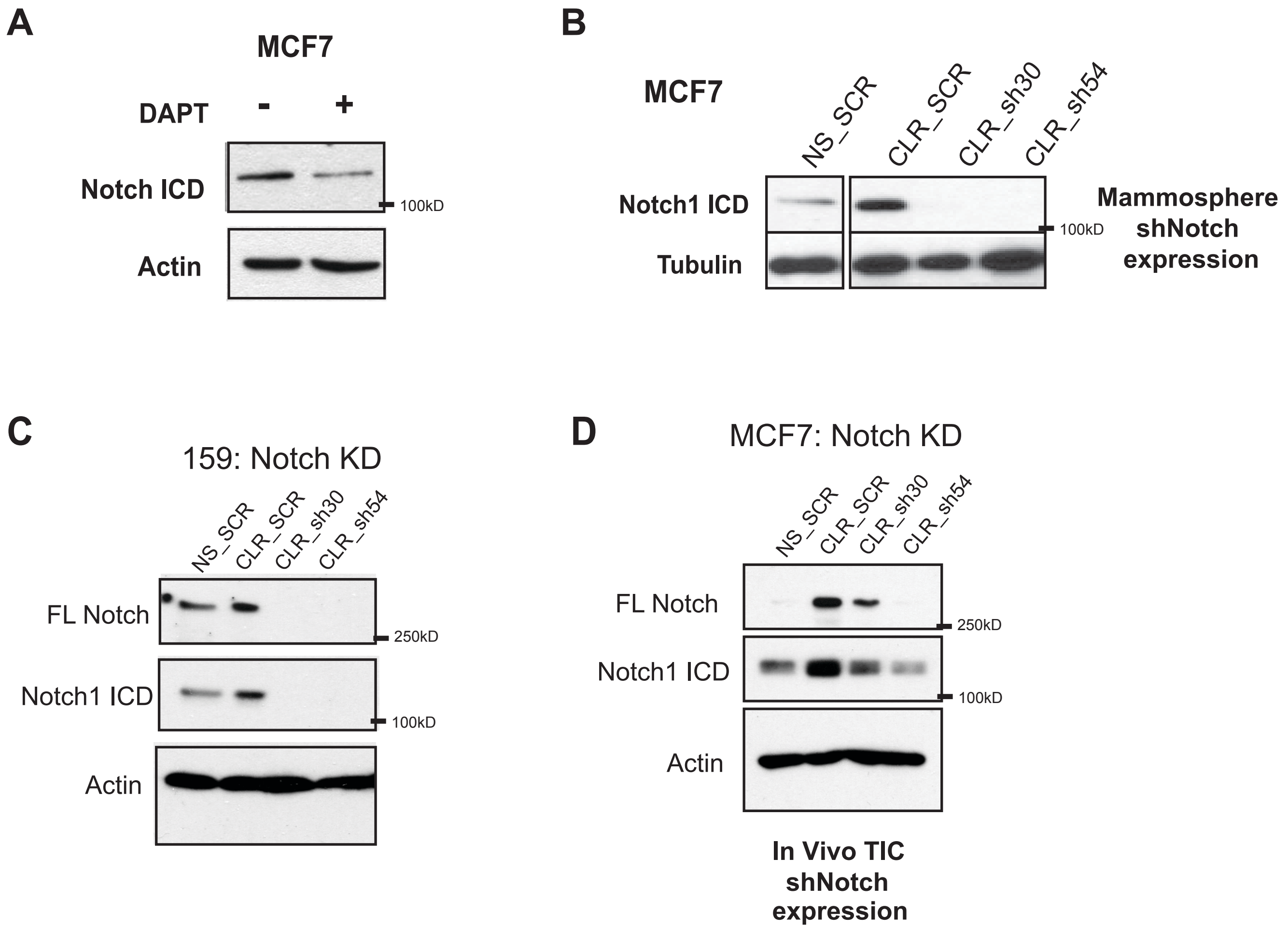


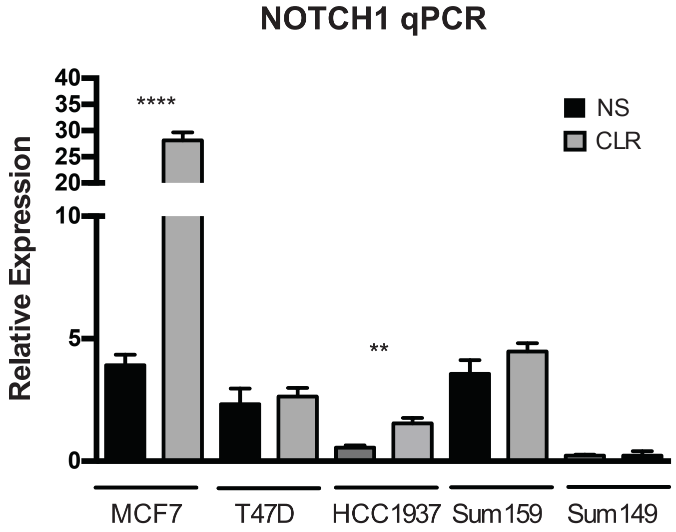
Supplemental Figure 1: Stable overexpression of the miR-106b-93 cluster of microRNAs. Quantitative real time RT-PCR in MCF7, T47D, HCC1937, Sum149, and Sum159 breast cancer cells for miR-106b, 93, 25 in the miR-106b-25 overexpressing (CLR) lines compared to the non-silencing (NS) lines. Results shown are from 3 replicate qRT-PCR reactions (+/- SD) normalized to U6 RNA.



Supplemental Figure 2: Individual miR-106b-25 mimics induce NOTCH1 overexpression. Western blot analysis of NOTCH1-ICD and full length (FL) NOTCH1 in MCF7 cells at 24hrs post transfection of 20nM miRNA mimics (Neg, miR-106b, miR-93, miR-25, and all three miRNAs together).

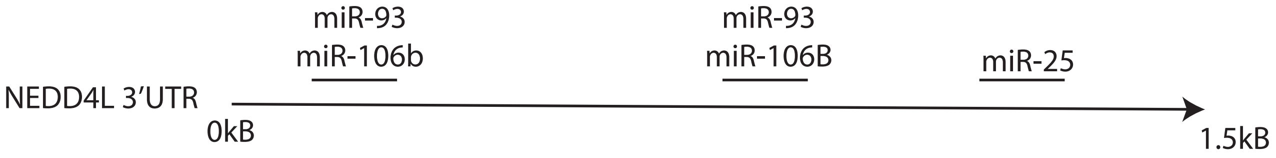


Supplemental Figure 3: Levels of Notch1 in drug and NOTCH1 KD treated cells. (A) Western blot analysis in MCF7 parental cells after treatment with the gamma-secretase inhibitor, DAPT (5uM). (B) Western blot analysis of MCF7 NOTCH1 KD cells showing efficient knock-down with both *NOTCH1* shRNAs (sh30, sh54) at the time of Mammosphere assays in MCF7 cells. (C) Analysis of NOTCH1 KD in Sum159 cell at the time of mammosphere formation assays showing efficient KD of both FL-NOTCH1 and NOTCH1-ICD protein forms. (D) Western blot analysis of NOTCH1 KD MCF7 cells at the time of the *in vivo* TIC experiments, showing a slight loss of NOTCH1 knock-down in sh30 containing cells, and also demonstrating efficient loss of FL-NOTCH1 .



NOTCH1 qPCR

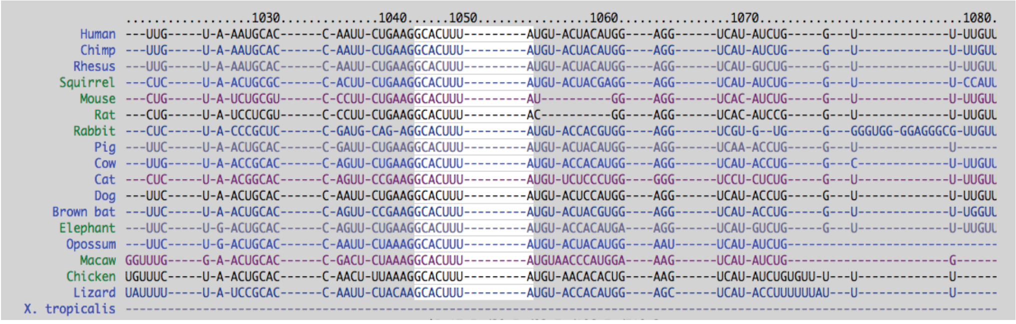
Supplemental Figure 4: miR-106b-25 overexpression does not increase *NOTCH1* mRNA in most cell lines. qRT-PCR in MCF7, T47D, HCC1937, Sum149, and Sum159 NS and CLR overexpressing cells for Notch1. Data shown are from 3 replicate qRT-PCR reactions (+/-) SD and normalized to GAPDH mRNA.



miR-93/miR-106b site 1

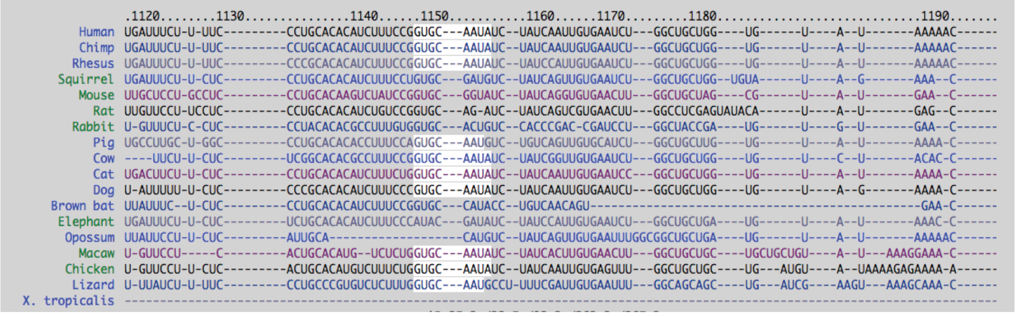
```
            ..................................40........50..............60............70..................80.................90.......
      Human ------------------------AAGC--AAGUUCUGCUUGCACU-----UUUG-CA-UUUGCCUAAC--A---GA------CUUU---U-GCAGAGGC----GAUGG---C--AG-A-GAGC
      Chimp ------------------------AAGC--AAGUUCUGCUUGCACU-----UUUG-CA-UUUGCCUAAC--A---GA------CUUU---U-GCAGAGGC----GAUGG---C--AG-A-GAGC
     Rhesus ------------------------AAGC--AAGUUCUGCUUGCACU-----UUUG-CA-UUUGCCUAAC--A---GA------CUUU---U-GCAGAGGC----GACGG---C--AG-A-GAGC
   Squirrel ------------------------AAGC--AAGUUCCCCUUGCACU-----UUUG-CA-UUUGCCUAAC--A---GA------C-UU---U-GCAGAGCA----GGGGG---G--AG---AGAC
      Mouse ------------------------CAGC--AAGUUCCAAGCGCACU-----UUUG-CA-UUUGCCGAGC--A---GA------CUUU---U-GCAGAGCC----GGUGG---C--AG---AAGC
        Rat ------------------------CAGC--AAGGUCCGCGUGCACU-----UUUG-CA-UUUGCCUAGC--A---GA------CUUU---U-GCAGAGCC----CGUGG---C--AG---AAGC
     Rabbit ------------------------GGGC--AAGUUCCGCUUGCACU-----UUCG-CG-UUGGCCUAAC--G---GA------C-UU---U-GCAGAGAC----GGUGG---CAGAG---CAGC
        Pig ------------------------GAGC--AAGUCCUGCUUGCACU-----UUUG-CA-UUUGCCUAAC--A---GA------C-UC---U-GCAGAGAC----CGUGG---C--AG-A-GAGC
        Cow ------------------------GAGC--AAGUCCUGCUCGCGCU-----UUUC-CA-UUUGCCUGAC--A---GA------C-UC---U-GCAGAGAC----GCUGG---C-----A-GAGC
        Cat ------------------------GAGC--GAGUCCUGCUUGCACU-----UUUG-CA-UUUGCCCAAC--ACCCGA------C-UU---U-GCAGAGAC----CGAGG---C--GGAA-GAGC
        Dog ------------------------CAGC--AAGUCCUGCUUGCACU-----UUUG-CA-UUUGCCUAAC--AACAGA------C-UU---U-GCAGAGAC----C-AGG---C--GGAG-GAGC
  Brown bat ------------------------CAGC--AAGUCCUGCUUGCACU-----UUUG-CG-UUUGCCUAAC--G---GA------C-UU---U-GCAGAGAC----GAUGG---C--AG-G-GAGC
   Elephant ------------------------GAGC--AAGUUCUGCUUGCACU-----UUUG-CG-UUUGCCUGAC--A---GA------CUUU---U-GCAGAGAC----GAUGG---C--GGAG-GCGC
    Opossum ------------------------AAGC--AAGUUUUUCUUGCCCU-----UUUG-CA-UUUGCCCAAU--G---GA------C--------ACAUAAGC----GACAA---U--GGCA-GAAC
      Macaw ------------------------GCAC--ACACUCUGCU--------------------UGGCCCGAG--G---G-------C--C---C-AUGGUGAC----UCUGG---C-----A-CAGC
    Chicken ------------------------GGGC--GCGUUCUGCUGC-GCU-----UCCA-CU-UCUCCACGAU--G---GA------C--U---C-----UGAC----UGUGG---C-----A-GAAC
     Lizard AAAAAAAUAACGAUUCUGUUCACGGAGC--AAGUUCUGCGUGCGCU-----UUUU-GA-UUUGCCCGAC--A---GA------C--AUUUG-CAAGAGGC----AGUGG---C-----A-GAAC
X. tropicalis ------------------------CAGCUAAAGCGCGGCUUGCACCGGGAACUUG-UG-CU------AC--G---GA------UUGU---A-CCACUGAC----AC--A---C--AA-G-GGGC
```

miR-93/miR-106b site 2



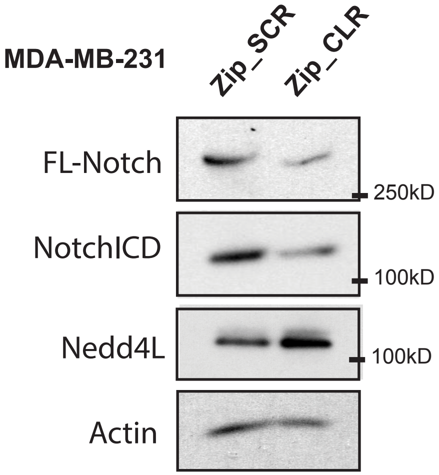
```
            ..................1030.............1040......1050...............1060................1070...........................1080.
      Human ---UUG-----U-A-AAUGCAC------C-AAUU-CUGAAGGCACUUU---------AUGU-ACUACAUGG---AGG------UCAU-AUCUG-----G---U-------------U-UUGUL
      Chimp ---UUG-----U-A-AAUGCAC------C-AAUU-CUGAAGGCACUUU---------AUGU-ACUACAUGG---AGG------UCAU-AUCUG-----G---U-------------U-UUGUL
     Rhesus ---UUG-----U-A-AAUGCAC------C-AAUU-CUGAAGGCACUUU---------AUGU-ACUACAUGG---AGG------UCAU-GUCUG-----G---U-------------U-UUGUL
   Squirrel ---CUC-----U-A-ACUGCGC------C-ACUU-CUGAAGGCACUUU---------AUGU-ACUACGAGG---AGG------UCAU-AUCUG-----G---U-------------U-CCAUL
      Mouse ---CUG-----U-A-UCUGCGU------C-CCUU-CUGAAGGCACUUU---------AU----------GG---AGG------UCAC-AUCUG-----G---U-------------U-UUGUL
        Rat ---CUG-----U-A-UCCUCGU------C-CCUU-CUGAAGGCACUUU---------AC----------GG---AGG------UCAC-AUCCG-----G---U-------------U-UUGUL
     Rabbit ---CUC-----U-A-CCCGCUC------C-GAUG-CAG-AGGCACUUU---------AUGU-ACCACGUGG---AGG------UCGU-G--UG-----G---GGGUGG-GGAGGGCG-UUGUL
        Pig ---UUC-----U-A-ACUGCAC------C-GAUU-CUGAAGGCACUUU---------AUGU-ACUACAUGG---AGG------UCAA-ACCUG-----G---U-------------U------
        Cow ---UUG-----U-A-ACCGCAC------C-AGUU-CUGAAGGCACUUU---------AUGU-ACCACAUGG---AGG------UCAU-ACCUG-----G---C-------------U-UUGUL
        Cat ---CUC-----U-A-ACGGCAC------C-AGUU-CCGAAGGCACUUU---------AUGU-UCUCCCUGG---GGG------UCCU-CUCUG-----G---U-------------U-UUGUL
        Dog ---UUC-----U-A-ACUGCAC------C-AAUU-CUGAAGGCACUUU---------AUGU-ACUCCAUGG---AGG------UCAU-ACCUG-----G---U-------------U-UUGUL
  Brown bat ---UUC-----U-A-ACUGCAC------C-AGUU-CCGAAGGCACUUU---------AUGU-ACUACGUGG---AGG------UCAU-ACCUG-----G---U-------------U-UGGUL
   Elephant ---UUC-----U-G-ACUGCAC------C-AGUU-CUGAAGGCACUUU---------AUGU-ACCACAUGA---AGG------UCAU-GUCUG-----G---U-------------U-UUGUL
    Opossum ---UUC-----U-G-ACUGCAC------C-AAUU-CUAAAGGCACUUU---------AUGU-ACUACAUGG---AAU------UCAU-AUCUG------------------------------
      Macaw GGUUUG----G-A-ACUGCAC------C-GACU-CUAAAGGCACUUU---------AUGUAACCAUGGA---AAG------UCAU-AUCUG-----------------------G------
    Chicken UGUUUC----U-A-ACUGCAC------C-AACU-UUAAAGGCACUUU---------AUGU-AACACACUG---AAG------UCAU-AUCUGUGUU-U---U-------------U------
     Lizard UAUUUU----U-A-UCCGCAC------C-AAUU-CUACAAGCACUUU---------AUGU-ACCACAUGG---AGC------UCAU-ACCUUUUUUAU---U-------------U------
X. tropicalis ---------------------------------------------------------------------------------------------------------------------------
```

miR-25

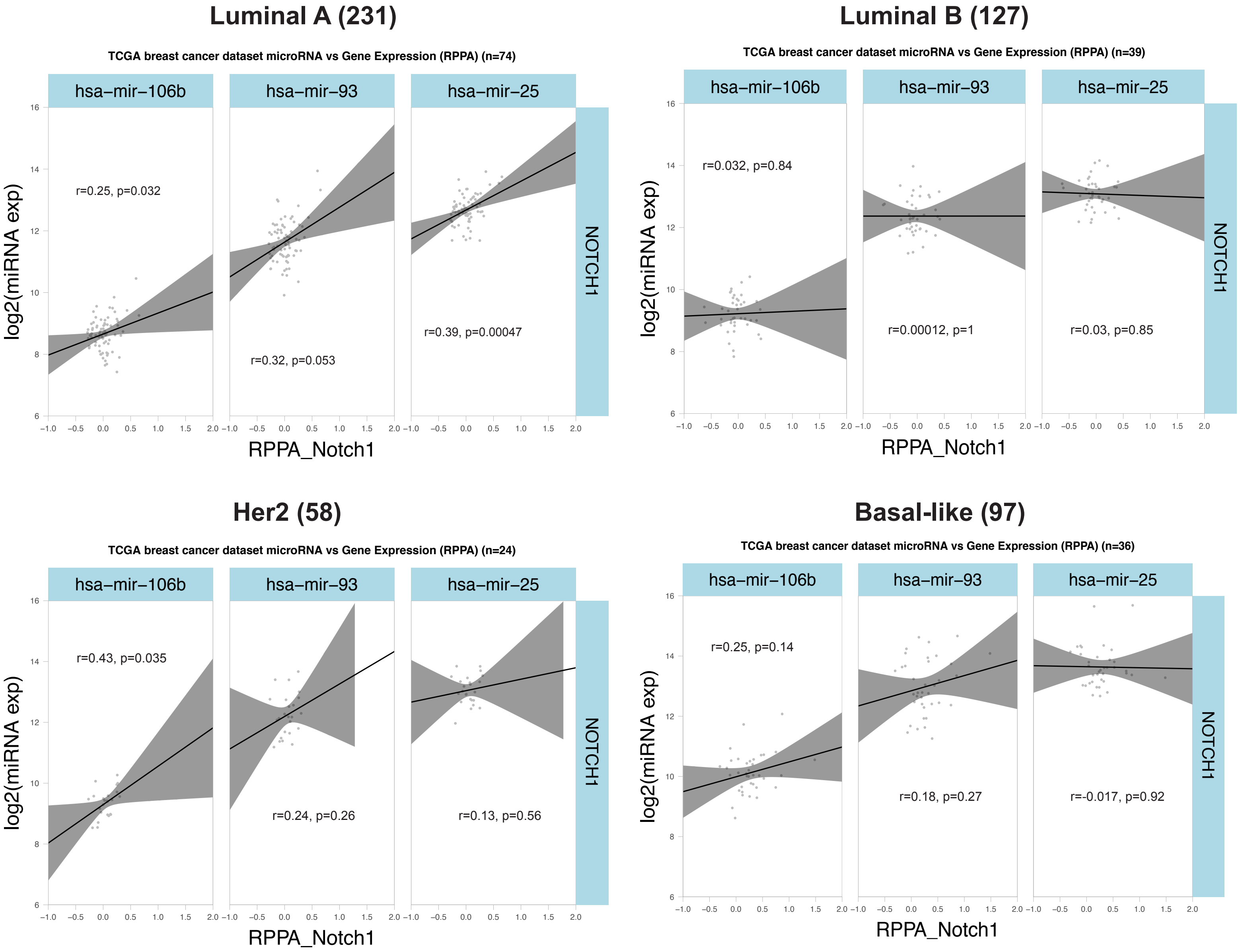


```
            .1120........1130...............1140......1150...........1160......1170.........1180............................1190.......
      Human UGAUUUCU-U-UUC---------CCUGCACACAUCUUUCCGGUGC---AAUAUC--UAUCAAUUGUGAAUCU---GGCUGCUGG----UG------U----A--U-------AAAAAC------
      Chimp UGAUUUCU-U-UUC---------CCUGCACACAUCUUUCCGGUGC---AAUAUC--UAUCAAUUGUGAAUCU---GGCUGCUGG----UG------U----A--U-------AAAAAC------
     Rhesus UGAUUUCU-U-UUC---------CCCGCACACAUCUUUCCGGUGC---AAUAUC--UAUCCAUUGUGAAUCU---GGCUGCUGG----UG------U----A--U-------AAAAAC------
   Squirrel UGAUUUCU-U-CUC---------CCUGCACACAUCUUUCCUGUGC---GAUGUC--UAUCAGUUGUGAAUCU---GGCUGCUGG--UGUA------U----A--G-------AAA--C------
      Mouse UUGCUCCU-GCCUC---------CCUGCACAAGUCUAUCCGGUGC---GGUAUC--UAUCAGGUGUGAACUU---GGCUGCUAG----CG------U----A--U-------GAA--C------
        Rat UUGUUCCU-UCCUC---------CCUGCACACAUCUGUCCGGUGC---AG-AUC--UAUCAGUCGUGAACUU---GGCCUCGAGUAUACA------U----A--U-------GAG--C------
     Rabbit U-GUUUCU-C-CUC---------CCUACACACGCCUUUGUGGUGC---ACUGUC--CACCCGAC-CGAUCCU---GGCUACCGA----UG------U----G--U-------GAA--C------
        Pig UGCCUUGC-U-GGC---------CCUGCACACACCUUUCCAGUGC---AAUGUC--UGUCAGUUGUGCAUCU---GGCUGCUUG----UG------U----A--U-------AAAA-C------
        Cow ----UUCU-U-CUC---------UCGGCACACGCCUUUCCGGUGC---AAUAUC--UAUCGGUUGUGAAUCU---GGCUGCUGG----UG------U----C--U-------ACAC-C------
        Cat UGACUUCU-U-CUC---------CCUGCACACAUCUUUCUGGUGC---AAUAUC--UAUCAAUUGUGAAUCC---GGCUGCUGG----UG------U----A--U-------AAAA-C------
        Dog U-AUUUUU-U-CUC---------CCCGCACACAUCUUUCCCGUGC---AAUAUC--UAUCAAUUGUGAAUCU---GGCUGCUGG----UG------U----A--G-------AAAA-C------
  Brown bat UUAUUUC--U-CUC---------CCUGCACACAUCUUUCCGGUGC---CAUACC--UGUCAACAGU-----------------------------------------------GAA-C------
   Elephant UGAUUUCU-U-CUC---------UCUGCACACAUCUUUCCCAUAC---GAUAUC--UAUCCAUUGUGAAUCU---GGCUGCUGA----UG------U----A--U-------AAAC-C------
    Opossum UUAUUCCU-U-CUC---------AUUGCA-------------------CAUGUC--UAUCAGUUGUGAAUUUGGCGGCUGCUGA----UG------U----A--U-------AAAAAC------
      Macaw U-GUUCCU-----C---------ACUGCACAUG--UCUCUGGUGC---AAUAUC--UAUCACUUGUGAACUU---GGCUGCUGC----UGCUGCUGU----A--U---AAAGGAAA-C------
    Chicken U-GUUCCU-U-CUC---------ACUGCACAUGUCUUUCUGGUGC---AAUAUC--UAUCAAUUGUGAGUUU---GGCUGCUGC----UG---AUGU----A--UAAAAGAGAAAA-A------
     Lizard U-UUAUCU-U-UUC---------CCUGCCCGUGUCUCUUUGGUGC---AAUGCCU-UUUCGAUUGUGAAUUU---GGCAGCAGC----UG---AUCG----AAGU---AAAGCAAA-C------
X. tropicalis ----------------------------------------------------------------------------------------------------------------------------
```

Supplemental Figure 5: miR-106b-25 targeted binding sites in the *NEDD4L*-3'UTR are conserved. Conservation analysis for all three miR-106b-25 binding sites in the 3'UTR of *NEDD4L*, showing a large degree of conservation from Human to Lizard.



Supplemental Figure 6: NOTCH1 is decreased, and NEDD4L is increased, with knockdown of miR106b-25 in MDA-MB-231 cells. Western blot analysis of MDA-MB-231 cells in which the miR-106b-25 cluster has been knocked down (Zip-CLR) vs. a non-targeting control (Zip-SCR)**.**



Luminal A (231)

TCGA breast cancer dataset microRNA vs Gene Expression (RPPA) (n=74)

Luminal B (127)

TCGA breast cancer dataset microRNA vs Gene Expression (RPPA) (n=39)

Her2 (58)

TCGA breast cancer dataset microRNA vs Gene Expression (RPPA) (n=24)

Basal-like (97)

TCGA breast cancer dataset microRNA vs Gene Expression (RPPA) (n=36)

Supplemental Figure 7: miR-106b and miR-93 correlate with Notch1 protein across multiple breast cancer subtypes. Data from the TCGA analysis was separated into breast cancer subtypes and re-analyzed for correlation between each miRNA in the miR-106b-25 cluster and Notch1 protein data (RPPA). Normal-like breast cancers did not have a big enough sample size to analyze. *P* values derived from KS test.