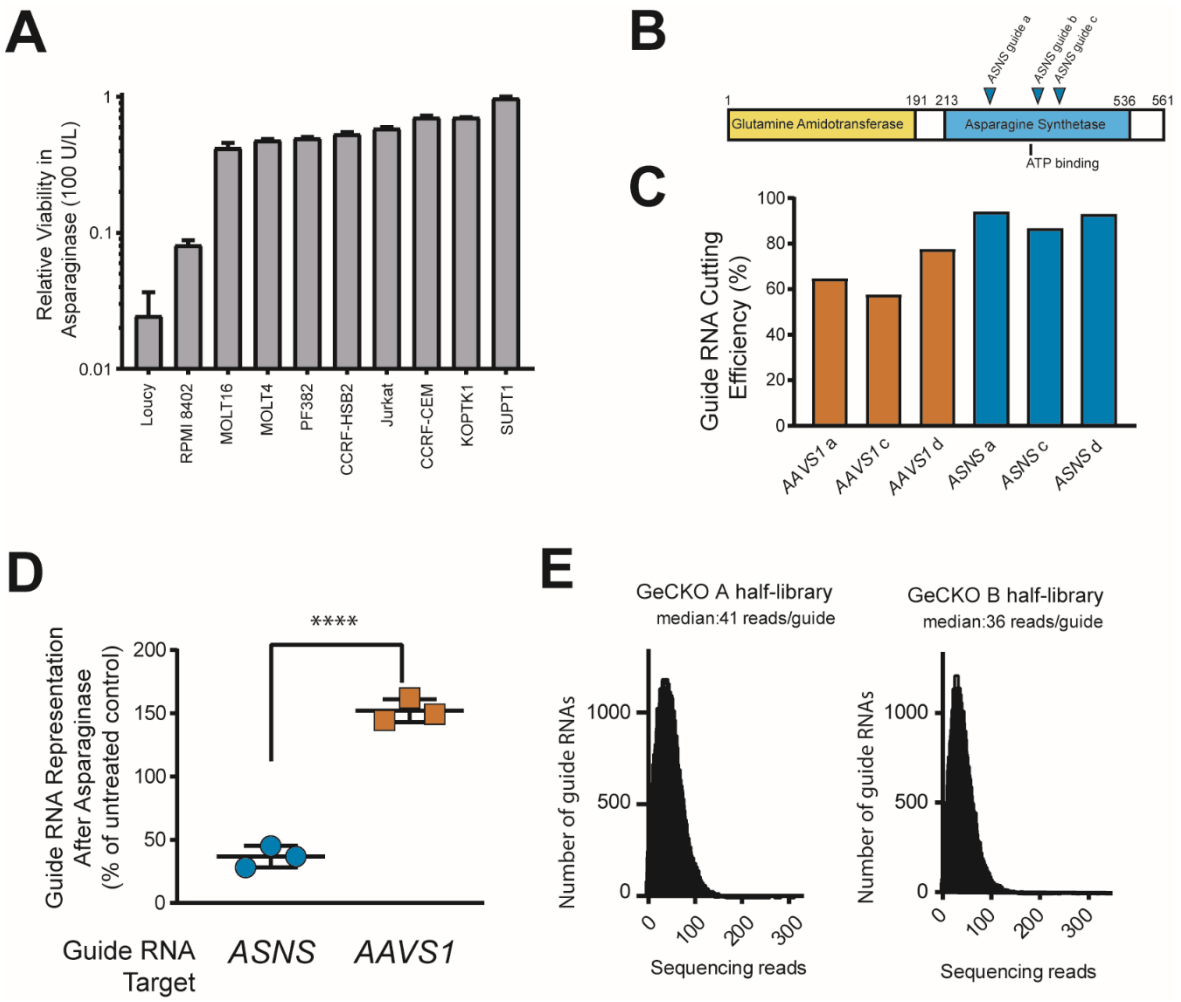

Figure S1, related to Figure 1. Optimization of a Screen for Asparaginase Sensitization in Human T-ALL Cell Lines.

(A) The indicated human T-ALL cell lines were treated with 100 U/L asparaginase or vehicle control for 48 hr, and viability was assessed by counting viable cells based on trypan blue vital dye staining. For each cell line, results were normalized to viability in vehicle-treated cells. Bars represent the mean of duplicate experiments, and error bars represent SEM. For the genetic screen, CCRF-CEM was selected because this was the most asparaginase-resistant T-ALL cell line in which we were able to achieve highly efficient CRISPR/Cas9 genome editing; we were unable to achieve detectable CRISRP/Cas9 genome editing in KOPTK1 or SUPT1 cells.

(B) Predicted location of the mutations induced by positive control guide RNAs targeting the asparagine synthetase catalytic domain. Colors indicate domains, and numbers indicate amino acids at the domain boundaries, based on the ASNS protein https://www.uniprot.org/uniprot/P08243.

(C) Cas9-expressing CCRF-CEM cells were transduced with positive control guide RNAs targeting the catalytic domain of asparagine synthetase (*ASNS*), or negative controls targeting the safe-harbor genomic locus *AAVS1*, and cutting efficiency was assessed by PCR amplification and next-generation sequencing of the target locus.

(D) Cas9-expressing CCRF-CEM cells were transduced with a pool of guide RNAs targeting *ASNS* or *AAVS1* (n = 3 guide RNAs targeting each locus), and subsequently treated with vehicle (PBS) or 10 U/L of asparaginase. After 5 days of treatment, guide RNA representation was assessed by next-generation sequencing. Abundance of each guide RNA was normalized to its abundance in vehicle-treated cells. Bars indicate the mean +/- SEM, and statistical significance was calculated using a Welch t-test.

(E) The GeCKO genome-wide guide RNA library, which is maintained in two distinct half-library pools (A and B), was amplified, and guide RNA representation with each half-library was assessed using next-generation sequencing.

All data are represented as mean +/- SEM. **** $p < 0.0001$. n.s., $p \geq 0.05$.

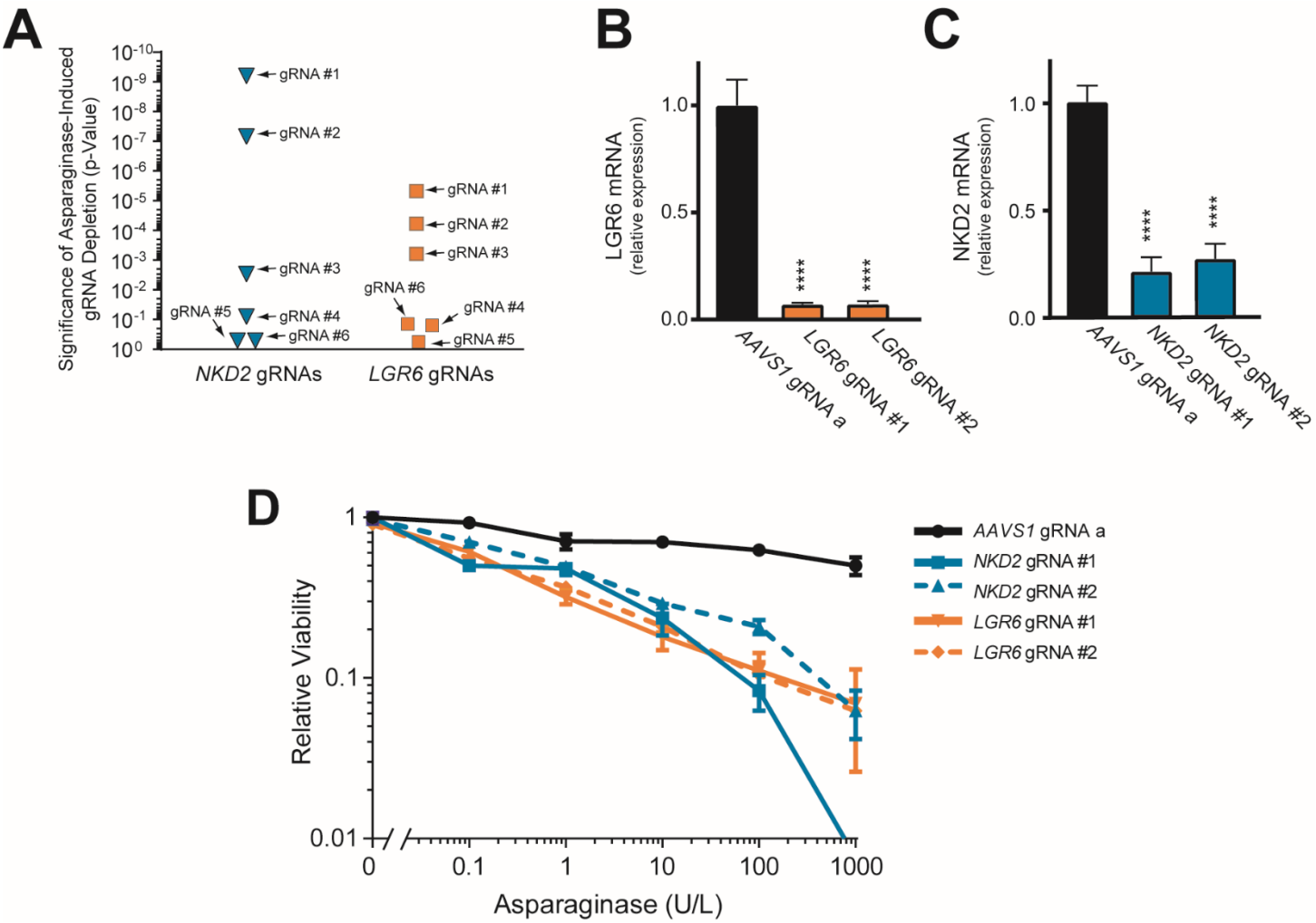

Figure S2, related to Figure 1. Individual guide RNAs targeting NKD2 or LGR6 Induce Sensitization to Asparaginase.

(A) Guide RNA (gRNA)-level assessment of significance of depletion in asparaginase-treated cells, as assessed by MAGeCK analysis. Each data point denotes significance of depletion for each individual guide RNA.

(B-C) Cas9-expressing CCRF-CEM cells were transduced with the two top-scoring gRNAs targeting *NKD2* or *LGR6,* and effects on mRNA expression levels were assessed by RT-PCR. Bars indicate the mean, and error bars indicate SEM.

(D) Cas9-expressing CCRF-CEM cells were transduced with the indicated gRNAs, treated with indicated doses of asparaginase, and viability was assessed after 8 days of treatment. Error bars indicate SEM.

**** $p < 0.0001$.

Table S2, related to Figure 1. Sequencing analysis of predicted off-target sites of top-scoring guide RNAs.

gRNA ID	off-targets for 0-1-2-3-4 mismatches	predicted off-target sites[1] (≤2 base pair mismatch)	% wild-type sequence at off-target site
NKD2 gRNA #1: HGLibA_32011	0-0-4-13-116	chr2:207,881,350-207,881,372	100
		chr7:121,172,772-121,172,794	100
		chr3:65,190,748-65,190,770	100
		chr16:57,777,630-57,777,652	100
NKD2 gRNA #2: HGLibA_32010	0-0-1-6-80	chr12:51,985,057-51,985,079	100
NKD2 gRNA #3: HGLibA_32009	0-0-0-2-6	none	not applicable
LGR6 gRNA #1: HGLibA_26413	0-0-0-8-71	none	not applicable
LGR6 gRNA #2: HGLibA_26414	0-0-0-8-76	none	not applicable
LGR6 gRNA #3: HGLibA_26415	0-0-1-10-149	chr21:39,289,056-39,289,078	100

[1]Genomic coordinates based on human genome build hg19

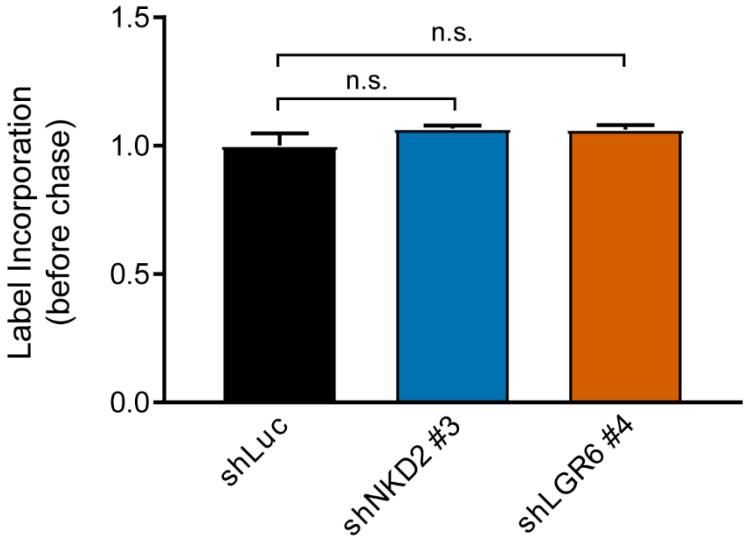

Figure S3, related to Figure 4. Wnt Pathway Activation Does Not Affect Degree of Label Incorporation During the Pulse.

CCRF-CEM cells transduced with indicated shRNAs were incubated with azidohomoalanine (AHA) for 18 hr. After the pulse, cells were subsequently fixed and fluorescence intensity of tagged AHA assessed by flow cytometry. Results shown are the mean of n = 3 biologic replicates, and error bars indicate SEM. Statistical significance was assessed by a one-way ANOVA with Dunnett's adjustment for multiple comparisons.

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. n.s., $p > 0.05$.

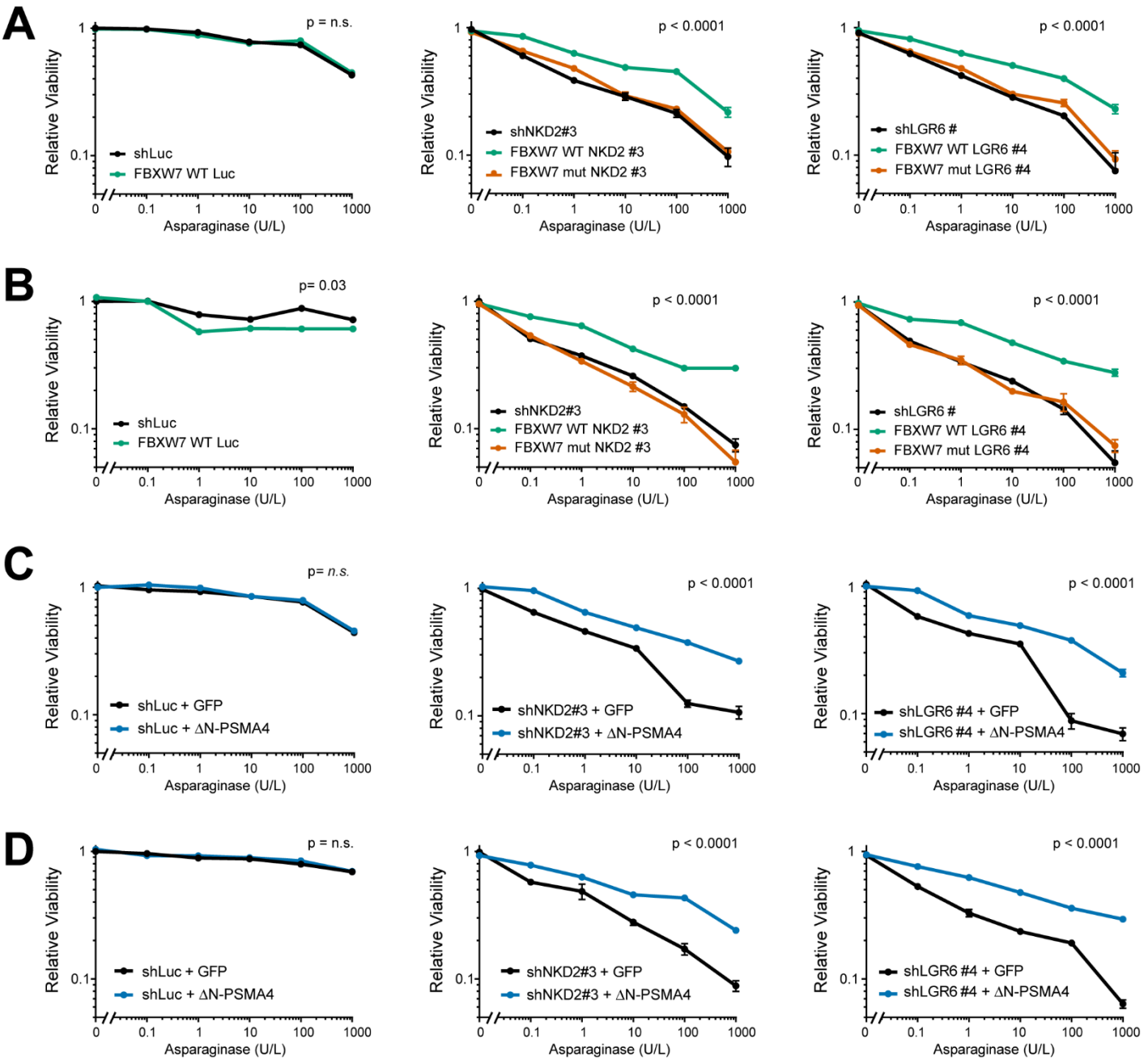

Figure S4, related to Figure 4. Overexpression of FBXW7 or Expression of the Hyperactive Proteasomal Subunit PSMA4 can Rescue Wnt-induced Sensitization to Asparaginase.

(A) CCRF-CEM cells were transduced with the indicated constructs and treated with the indicated doses of asparaginase for 8 days. Viability was assessed by counting trypan blue viable cells. All cell counts are normalized to those in shLuc-transduced, vehicle-treated cells.

(B) DND41 cells were transduced with the indicated constructs, treated with asparaginase, and viability was assessed as in (A).

(C) CCRF-CEM cells were transduced with the indicated constructs, treated with asparaginase and viability was assessed as in (A).

(D) DND41 cells were transduced with the indicated constructs, treated with asparaginase and viability was assessed as in (A).

Two-way ANOVA with Tukey post-hoc adjustment was performed for each cell line and included an interaction term between asparaginase dose and transduces construct. The p value for the main effect of GFP or DPSMA4 vs. vehicle is presented in each plot and all interaction terms were significant ($p < 0.0001$). All error bars indicate SEM.

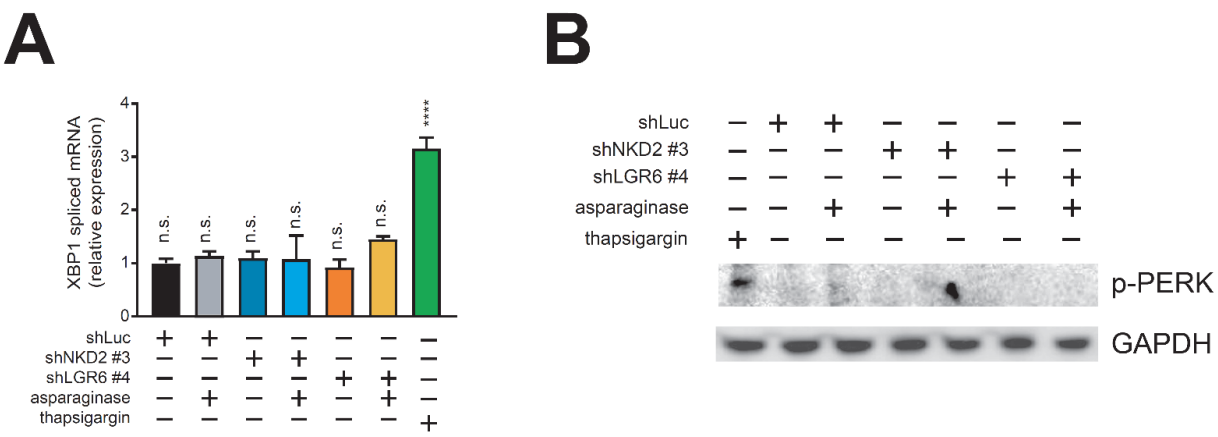

Figure S5, related to Figure 4. Wnt Pathway Activation and Asparaginase Do not Activate the Unfolded Protein Response.

(A) CCRF-CEM cells were transduced with the indicated constructs and treated with 10 U/L asparaginase or 100 nM thapsigargin. Effects on mRNA expression levels were assessed by RT-PCR**.** Error bars denote SEM. A one-way analysis of ANOVA with a Dunnett adjustment for multiple comparisons was used to test each group compared to the shLuc control. **** $p < 0.0001$. n.s., $p \geq 0.05$.

(B) CCRF-CEM cells were treated as indicated and protein lysates analyzed for phospho-PERK (Thr980) by Western blot.

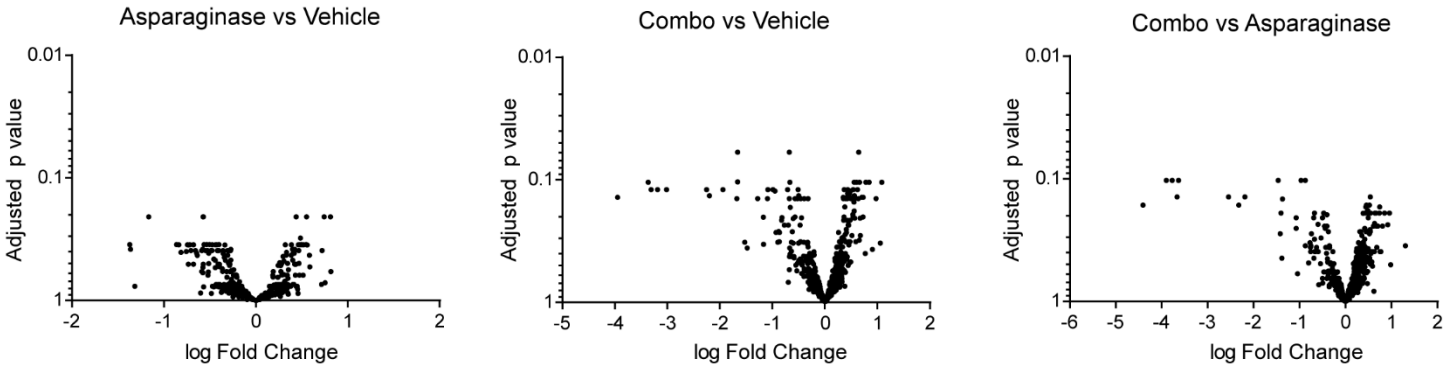

Figure S6, related to Figure 5. Wnt Pathway Activation and Asparaginase Do Not Have Profound Effects on Individual Proteins.

CCRF-CEM cells were treated with control, asparaginase (10 U/L), the GSK3α inhibitor BRD0705 (100 nM), or the combination of both drugs (combo). The caspase inhibitor Z-VAD-FMK (2 μM) was included in all conditions, because our goal was to detect causes, rather than consequences, of cell death. After 48 hr of treatment, proteins were extracted and mass spectrometry proteomics analysis performed. Differential expression analysis for the indicated comparisons was performed using the voom-limma method with an empirical Bayes statistic (eBayes(trend=TRUE, robust=TRUE)) in R, and p values were adjusted for multiple hypothesis testing using the method of Benjamini-Hochberg (or false discovery rate). Data are available via ProteomeXchange with identifier PXD013061.

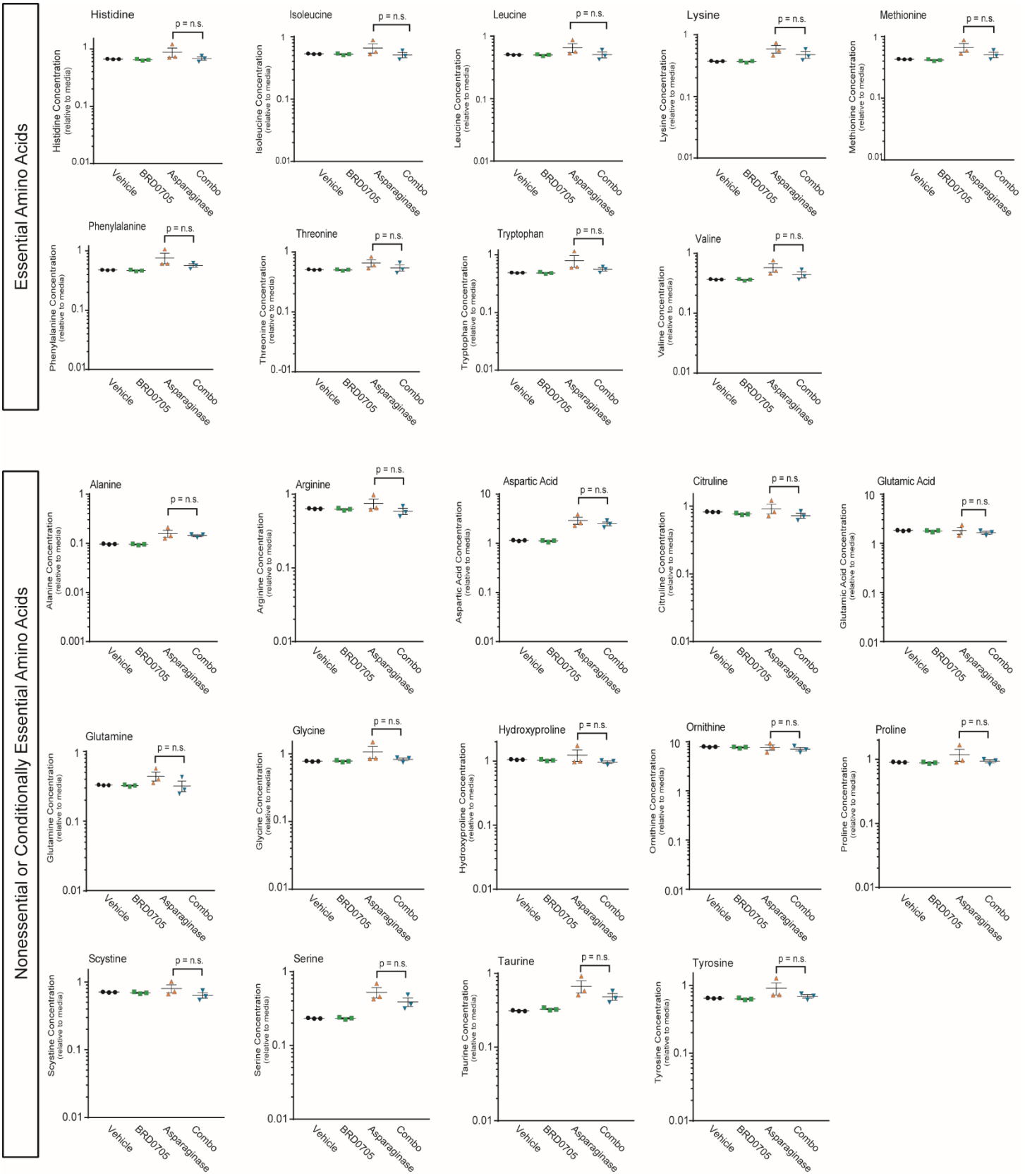

Figure S7, related to Figure 5. Amino acid quantification in CCRF-CEM cells. CCRF-CEM cells were treated with vehicle, asparaginase (10 U/L), the GSK3α inhibitor BRD0705 (100 nM) or both in combination (combo) for 12 hr. Culture media was collected and levels of the indicated amino acids were measured by UPLC. Stock media (RPMI-1640) was used as a control for each UPLC run, and amino acid levels are normalized to those in media. Differences between groups were assessed using a two-sided Welch t-test. Bars denote the mean, and error bars denote SEM.

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. n.s., $p > 0.05$.

Table S5, related to STAR Methods. Primer and guide RNA sequences.

Guide RNA	**Guide RNA Sequence (+ strand)**
ASNS gRNA #1	GGAAGACAGCCCCGATTTAC
ASNS gRNA #2	AGGATCAGATGAACTTACGC
ASNS gRNA #3	CAGCAGTAGTTCGATCTGCG
AAVS1 gRNA #1	AGCGGCTCCAATTCGGAAGT
AAVS1 gRNA #2	GAGAGGTGACCCGAATCCAC
AAVS1 gRNA #3	AGTTCTTAGGGTACCCCACG
LGR6 gRNA #1	GCGCCGCGCTGTGCGCTTCC
LGR6 gRNA #2	GTCCAGGTCCCCCGGAACGG
LGR6 gRNA #3	CTGATTTCCAGGCGTCTCTC
NKD2 gRNA #1	CAAGCGGAGAGAGAGCCCGG
NKD2 gRNA #2	CCGCTTCCTCCGCGCCTTTG
NKD2 gRNA #3	GCGTACGCGAGCGGCCGCAA
Primer Name	**Primer Sequence**
pHKO9 F	TCTTGTGGAAAGGACGAAACACCG
pHKO9 R	TCTACTATTCTTTCCCCTGCACTGT
Human b-actin F	CTGGCACCCAGCACAATG
Human b-actin R	GCCGATCCACACGGAGTACT
Human NKD2 F	ACCCTCCGTGTGAAGCTAAC
Human NKD2 R	GGGCCTCCTGACGTGTG
Human LGR6 F	TCGGGCTGTCCGCCGTTC
Human LGR6 R	GCTCCTCCAAGAAGCGCAGGTG
Human APC F	AACCGCTGCAGATGGCTGATGTG
Human APC R	TGCAGCCATCCTTGGCTACCCT
Human GSK3a F	TCCCCAGCGGGCACTACCA
Human GSK3a R	TGAGGGTAGGTGTGG CATCGGT
Human GSK3b F	CCAGGGGATAGTGGTGTGGATCAGT
Human GSK3b R	GAAGACCCGCACTCCTGAGCTGA
XBP1 spliced F	TGCTGAGTCCGCAGCAGGTG
aluXBP1 spliced R	GCTGGCAGGCTCTGGGGAAG
chr2:207,881,350-207,881,372 F	ATTGTCTCACAGTTCTGGAGGC
chr2:207,881,350-207,881,372 R	AAAGTGAGGACCCTAATCCAG
chr7:121,172,772-121,172,794 F	ATTGGCTAAAGTTTCAGCAGAC
chr7:121,172,772-121,172,794 R	GAAAGGATGGCCACTCCAAC
chr3:65,190,748-65,190,770 F	ATTTCACTCCCATTGAACGTGGC

chr3:65,190,748-65,190,770 R	TATGGTCTTTCCAGTATGGTGG
chr21:39,289,056-39,289,078 F	GGAATCTTTTGGGTTTCGGTC
chr21:39,289,056-39,289,078 R	CCCAGAGCCTCTTTTTCCTTAT
chr16:57,777,630-57,777,652 F	AGGCTTGTTTTAGAACGCC
chr16:57,777,630-57,777,652 R	TCACTGTGGATCCTCTATCT
chr12:51,985,057-51,985,079 F	CAATTATGCAAGGACTCGGGC
chr12:51,985,057-51,985,079 R	CAAAGCCCCTGACTGGAGAT
Illumina F1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAGTAGAGT CTTGTGGAAAGGACGAAACACCG
Illumina F2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATACACGATC TCTTGTGGAAAGGACGAAACACCG
Illumina R1	CAAGCAGAAGACGGCATACGAGATAAGTAGAG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTCTACTATTCTTTCCCCTGCACTGT
Illumina R2	CAAGCAGAAGACGGCATACGAGATACACGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTA TTCTACTATTCTTTCCCCTGCACTGT
Illumina R3	CAAGCAGAAGACGGCATACGAGATCGCGCGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT GATTCTACTATTCTTTCCCCTGCACTGT
Illumina R5	CAAGCAGAAGACGGCATACGAGATCGTTACCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTT CGATTCTACTATTCTTTCCCCTGCACTGT
Illumina R6	CAAGCAGAAGACGGCATACGAGATTCCTTGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTA TCGATTCTACTATTCTTTCCCCTGCACTGT