Journal: Nature Medicine

Supplementary Information

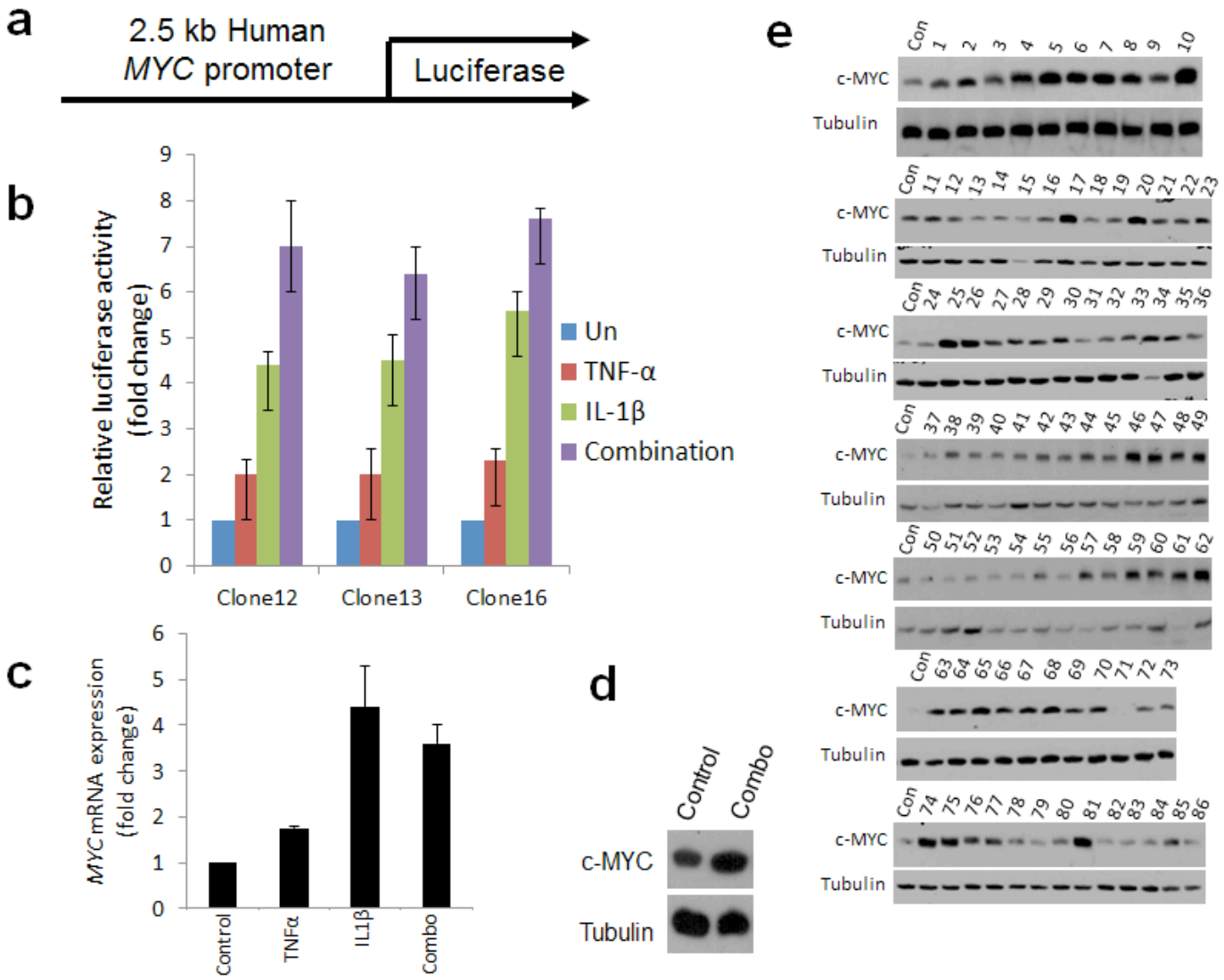
Induction of human pancreatic beta cell replication by inhibitors of dual specificity tyrosine regulated kinase

[1,2]Peng Wang PhD, [1,2]Juan-Carlos Alvarez-Perez PhD, [3,4]Dan P. Felsenfeld PhD,
[1]Hongtao Liu BA, [3,4]Sharmila Sivendran PhD, [1,2]Aaron Bender BS,
[1,2]Anil Kumar PhD, [3]Roberto Sanchez PhD, [1,2,5]Donald K. Scott PhD,
[1,2,5]Adolfo Garcia-Ocaña PhD, [1,2,6]Andrew F. Stewart MD

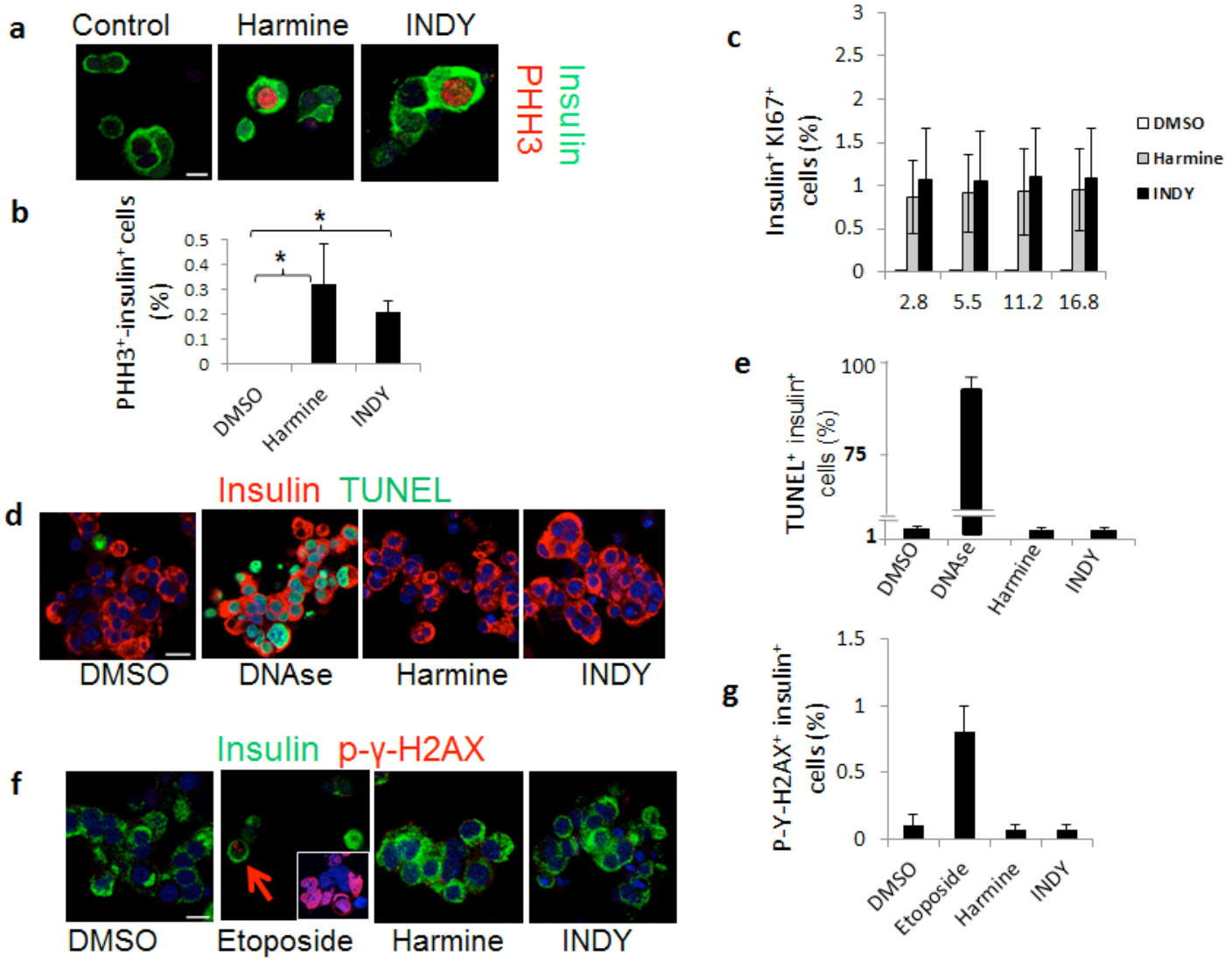
From: [1]The Diabetes, Obesity and Metabolism Institute, Icahn School of Medicine at Mount Sinai, NY, NY USA
[2]The Division of Endocrinology and Bone Disease, Icahn School of Medicine at Mount Sinai, NY, NY USA
[3]The Experimental Therapeutics Institute, Icahn School of Medicine at Mount Sinai, NY, NY USA
[4]The Integrated Screening Core, Icahn School of Medicine at Mount Sinai, NY, NY USA
[5]The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, NY, NY USA

1. Supplementary Figures
2. Supplementary Table 1: PCR Primers

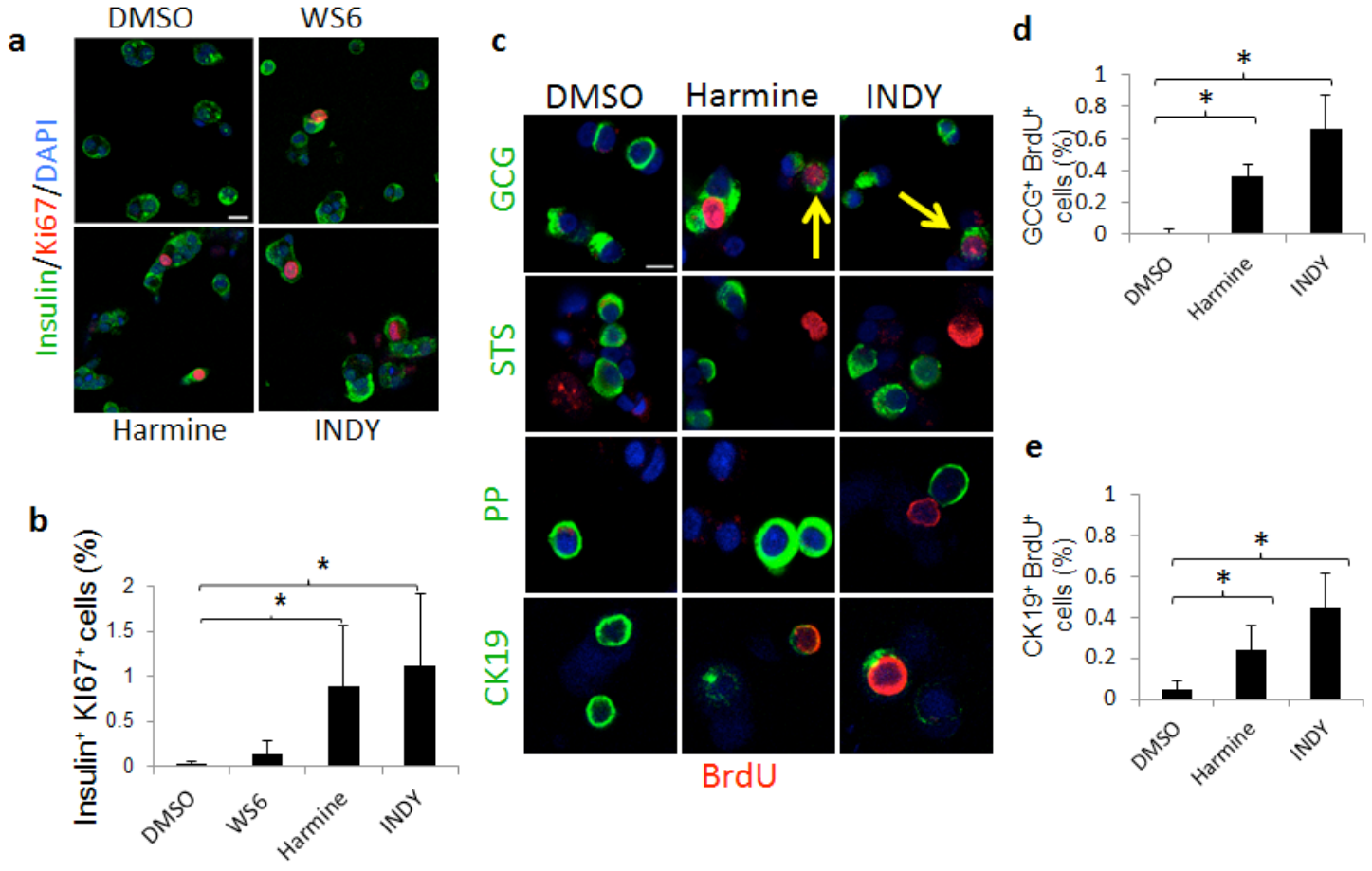
Supplementary Figures.



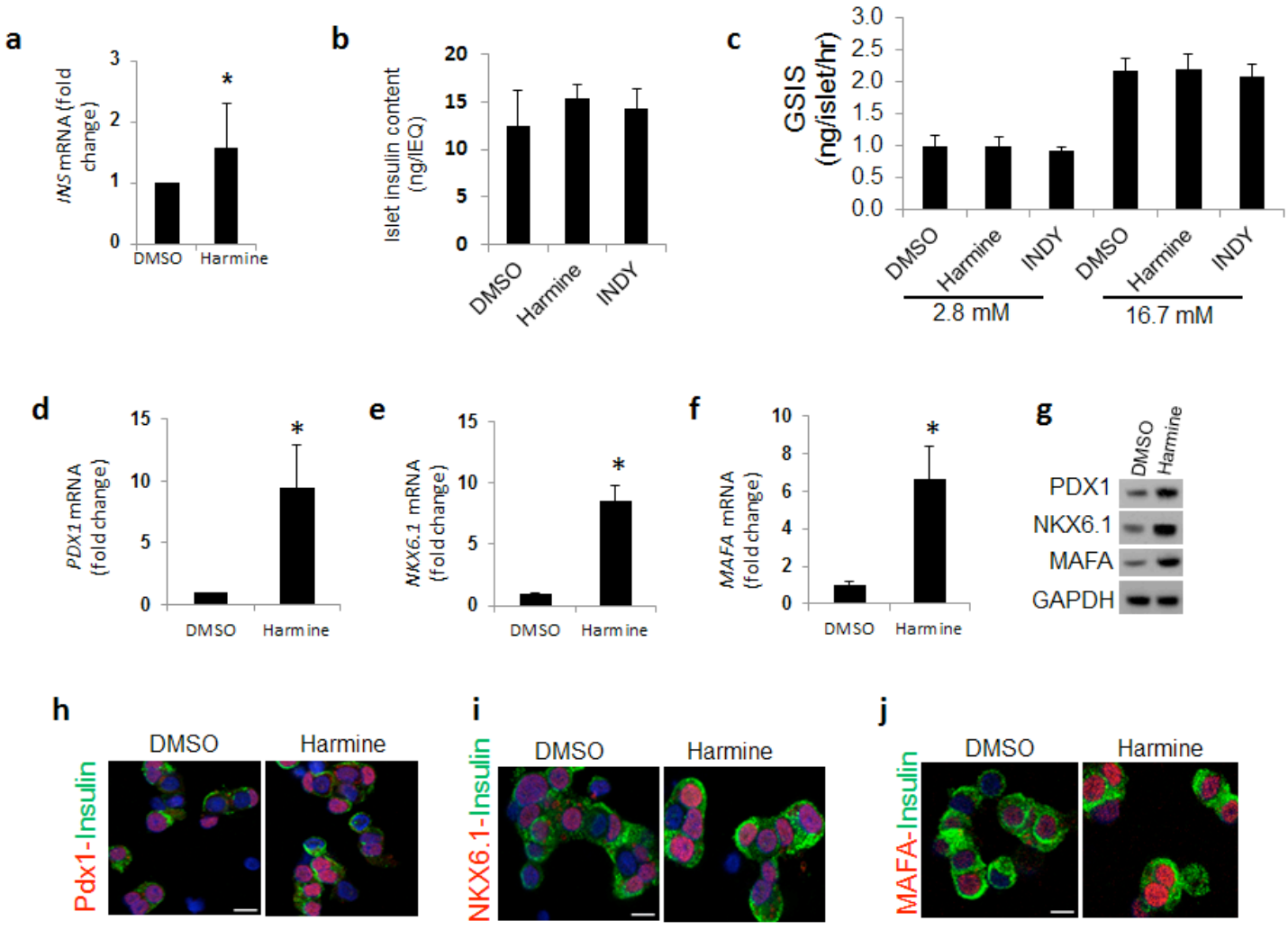
Supplementary Figure 1. The strategy and validation of methods for the high-throughput screen (HTS). (**a**) The *MYC* promoter-driven luciferase reporter construct. (**b**) Examples of HepG2-*MYC* -luciferase stable clones responding to the IL-1β or TNF-α or a combination (Combo). The cytokine combination was selected because the *MYC* promoter has NFkB sites. Clone 13 was selected for the HTS. (**c**) Endogenous *MYC* mRNA responses in HepG2 cells to IL-1β and TNF-α for 8 hr. Bars denote mean ± s.e.m. (**d**) A representative immunoblot of the c-MYC protein response to IL-1β and TNFα 10 ng ml^{-1} in HepG2 cells. (**e**) Secondary screening for c-MYC by immunoblot in HepG2 cells of all 86 compounds meeting criteria in the primary luciferase screen. "Con" indicates vehicle (DMSO). Harmine is Compound 1.



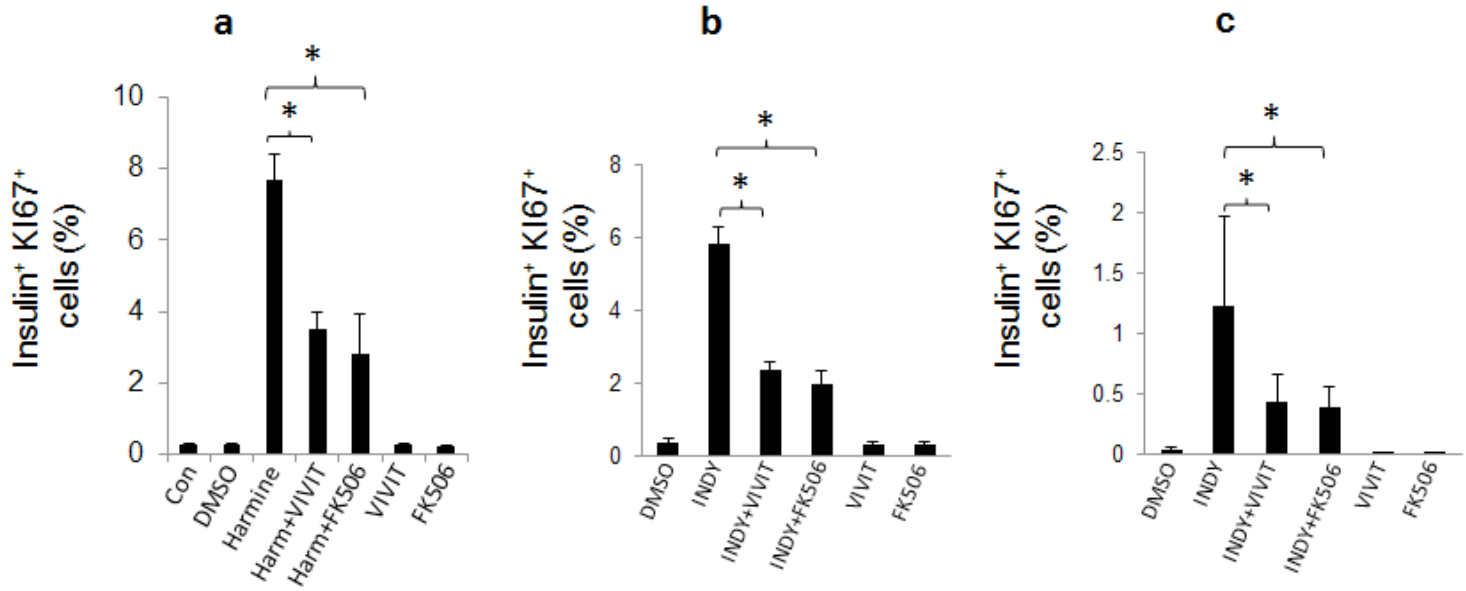
Supplementary Figure 2. Effects of harmine or INDY on beta cell PHH3, DNA damage and survival. (**a**) Examples of harmine (10 μM)- or INDY (15 μM)-treated (four days) dispersed human islets immunolabeled using the G2/M marker, phospho-histone-3 (PHH3). (**b**) Quantification of the percent insulin$^+$/PHH3$^+$ cells. Four different human islet preparations were studied. (**c**) Effects of glucose concentrations on Ki67 labeling in response to harmine. Four different human islet preparations were studied. (**d**) TUNEL labeling in human islets treated for three days with harmine or INDY as in (**a**). DNase-treated islets served as positive control. (**e**) Quantification of TUNEL labeling in three different human islet preparations. (**f**) Examples of the effects of harmine or INDY treatment as in (**a**) on DNA damage in beta cells as detected by immunolabeling for the DNA damage marker, phospho-γ-H2AX in response to etoposide treatment in beta cells. The inset in the etoposide panel includes HEK293 cells that were used as positive control, since non-replicating beta cells do not display DNA damage in response to etoposide. The blue signal is DAPI. (**g**) Quantification of phospho-γ-H2AX in beta cells in four different human islet preparations. In all relevant panels, error bars indicate mean ± s.e.m.; the scale bar indicates 10 μm; * indicates $P<0.05$ as determined by Wilcoxon Rank test.



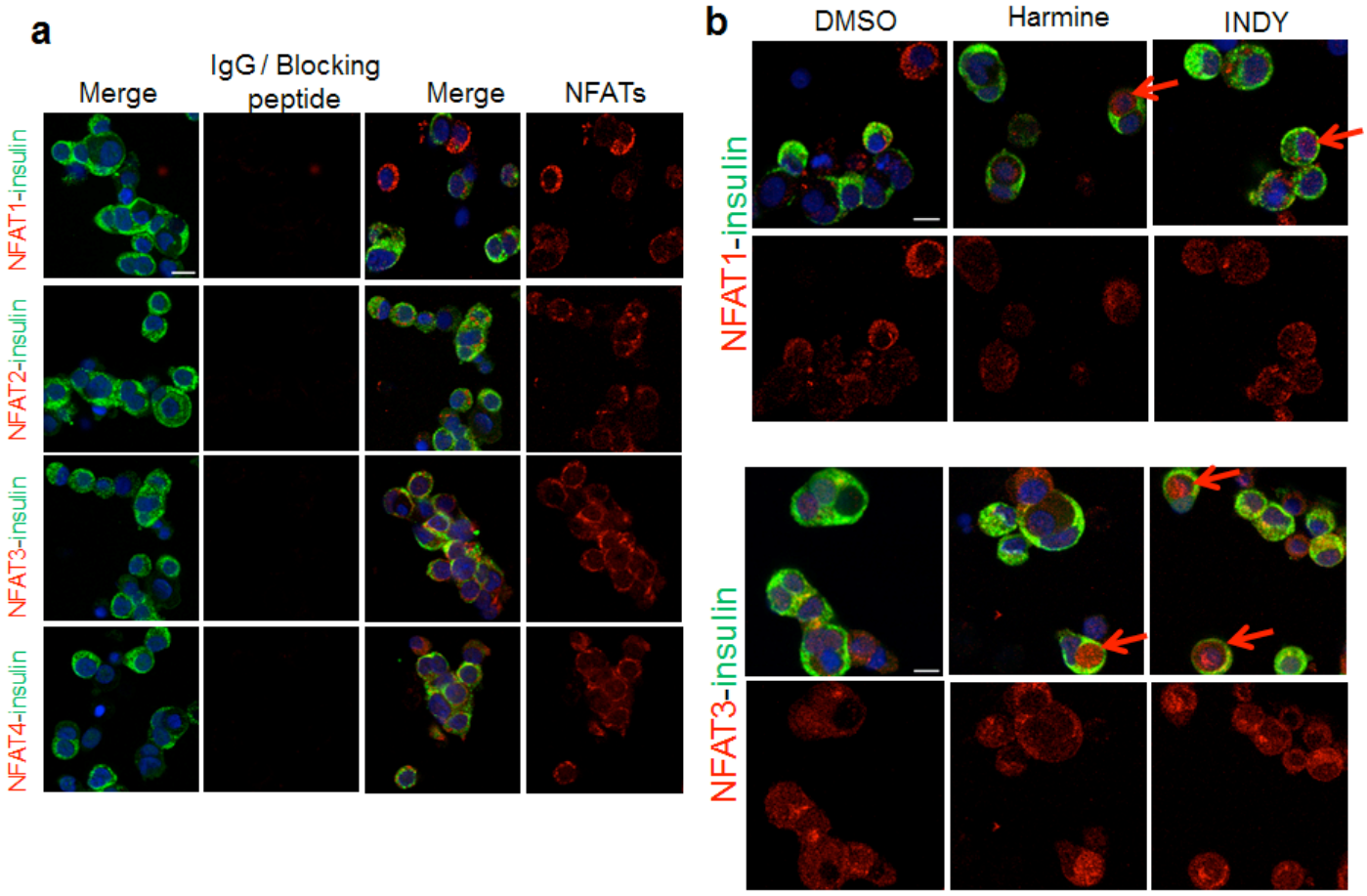
Supplementary Figure 3. Harmine and INDY vs. WS6 and human beta cell proliferation, and proliferation in non-beta islet cells. (**a**) Examples of dispersed human islets treated with 1 μM WS6, 10 μM Harmine, or 15 μM INDY for four days, and immunolabeled for Ki67 and insulin. (**b**) Quantification of beta cell Ki67 labeling in response to WS6, harmine, or INDY in seven human islet preparations. A minimum of 1000 beta cells was counted for each bar. (**c**) Examples of BrdU labeling in non-beta islet cells in human islets treated with DMSO (vehicle), 10 µM harmine or 15 µM INDY, and immunolabeled for BrdU, glucagon (GCG), somatostatin (STS), pancreatic polypeptide (PP) or the ductal cell marker CK19. (**d**) Quantification of proliferation in alpha cells (GCG) and (**e**) ductal (CK19) cells in four different sets of human islets. In all relevant panels, bars indicate s.e.m.; * indicates P<0.05 as determined by Wilcoxon Rank test; the scale bar indicates 10 µm.



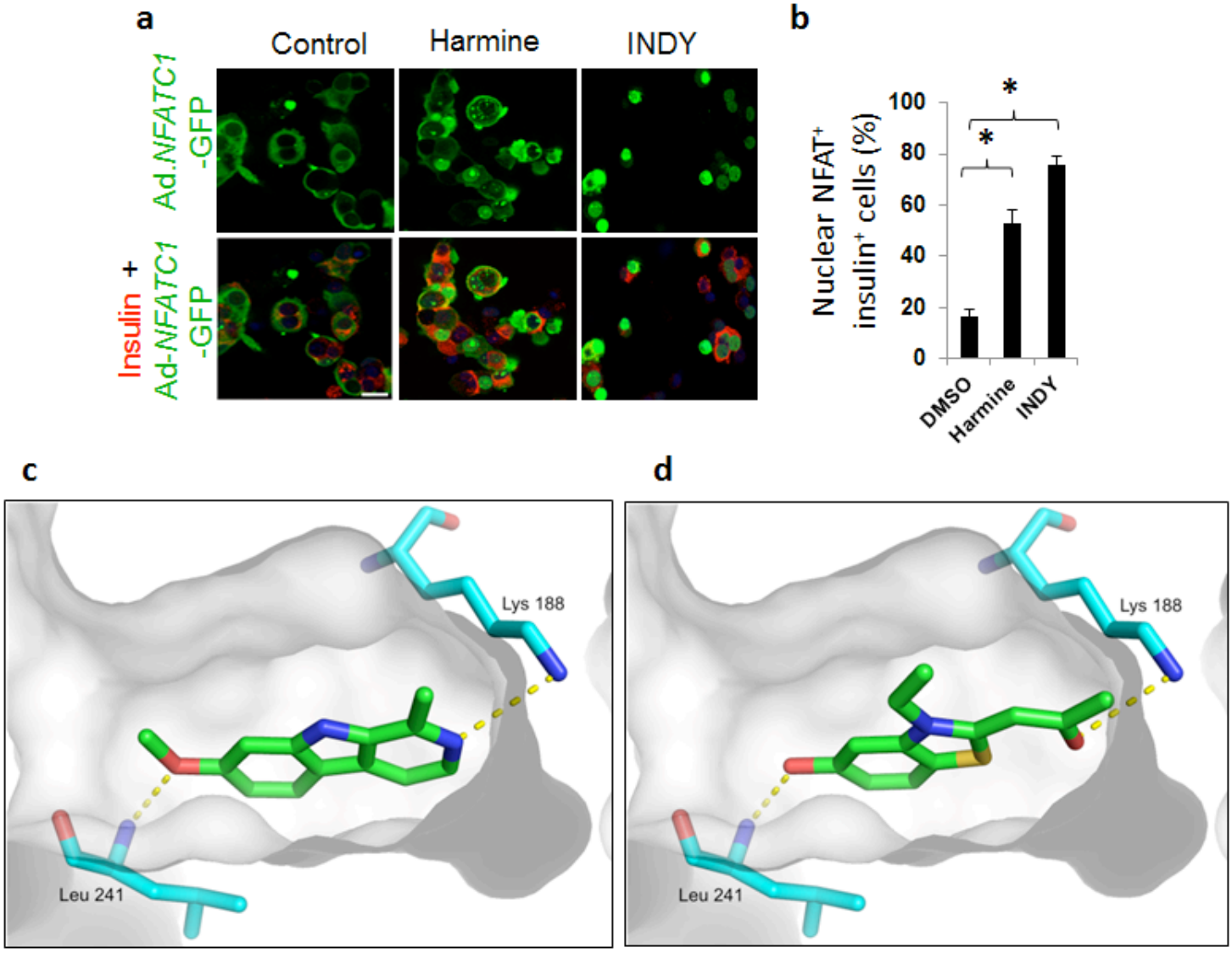
Supplementary Figure 4. Effects of Harmine and INDY on markers of beta cell differentiation. (**a**) *INS* mRNA expression by qPCR in four different human islet preparations treated with DMSO or harmine (10 μM) for 72 hours. (**b**) Effects of DMSO or harmine as in (**a**) on human islet insulin content in five different human islet preparations. (**c**) Effects of DMSO or harmine treatment as in (**a**) on glucose-stimulated insulin secretion from human islets. (**d**–**f**) Effects of DMSO or harmine treatment as in (**a**) on human islet *PDX1*, *NKX6.1* and *MAFA* mRNA expression in four different human islet preparations. (**g**) Representative examples of immunoblots for PDX1, NKX6.1 and MAFA in human islets treated with harmine as above. Three different human islet preparations were examined for each transcription factor. (**h**–**j**) Representative examples of immunocytochemistry for PDX1, NKX6.1 and MAFA in three sets of dispersed human islets treated with vehicle or harmine (10 μM for 72 hours). In all relevant panels, error bars indicate s.e.m.; *indicates $P<0.05$ as determined by Wilcoxon Rank test; the scale bar indicates 10 μm.



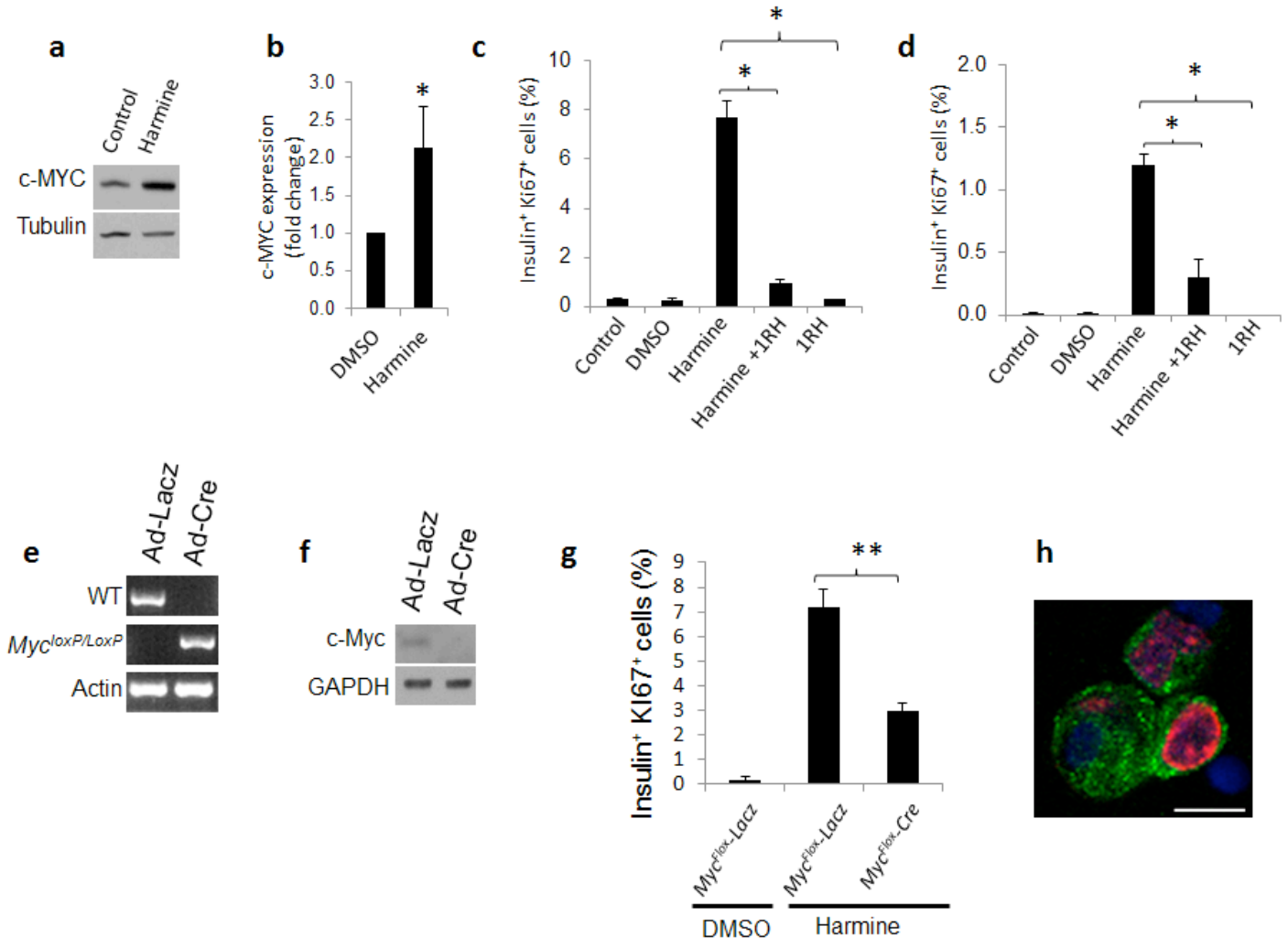
Supplementary Figure 5. Calcineurin-NFAT-DYRK1A signaling in harmine- and INDY-induced beta cell proliferation. (**a**) Effects of harmine (10 μM, 72 hr) on rat beta Ki67 labeling, and of inhibition with VIVIT (2.5 μM) or FK506 (10 μM) on four different rat islet preparations. (**b**)**.** Effects of INDY (15 μM, 72 hr) on rat beta Ki67 labeling, and inhibition with VIVIT or FK506 as in (**a**) on four different rat islet preparations. (**c**) Effects of INDY (15 μM, 72 hr) on human beta Ki67 labeling, and inhibition with VIVIT or FK506 as in (**a**) on four different human islet preparations. In all panels, error bars indicate s.e.m.; *indicates $P<0.05$ as determined by Wilcoxon Rank test.



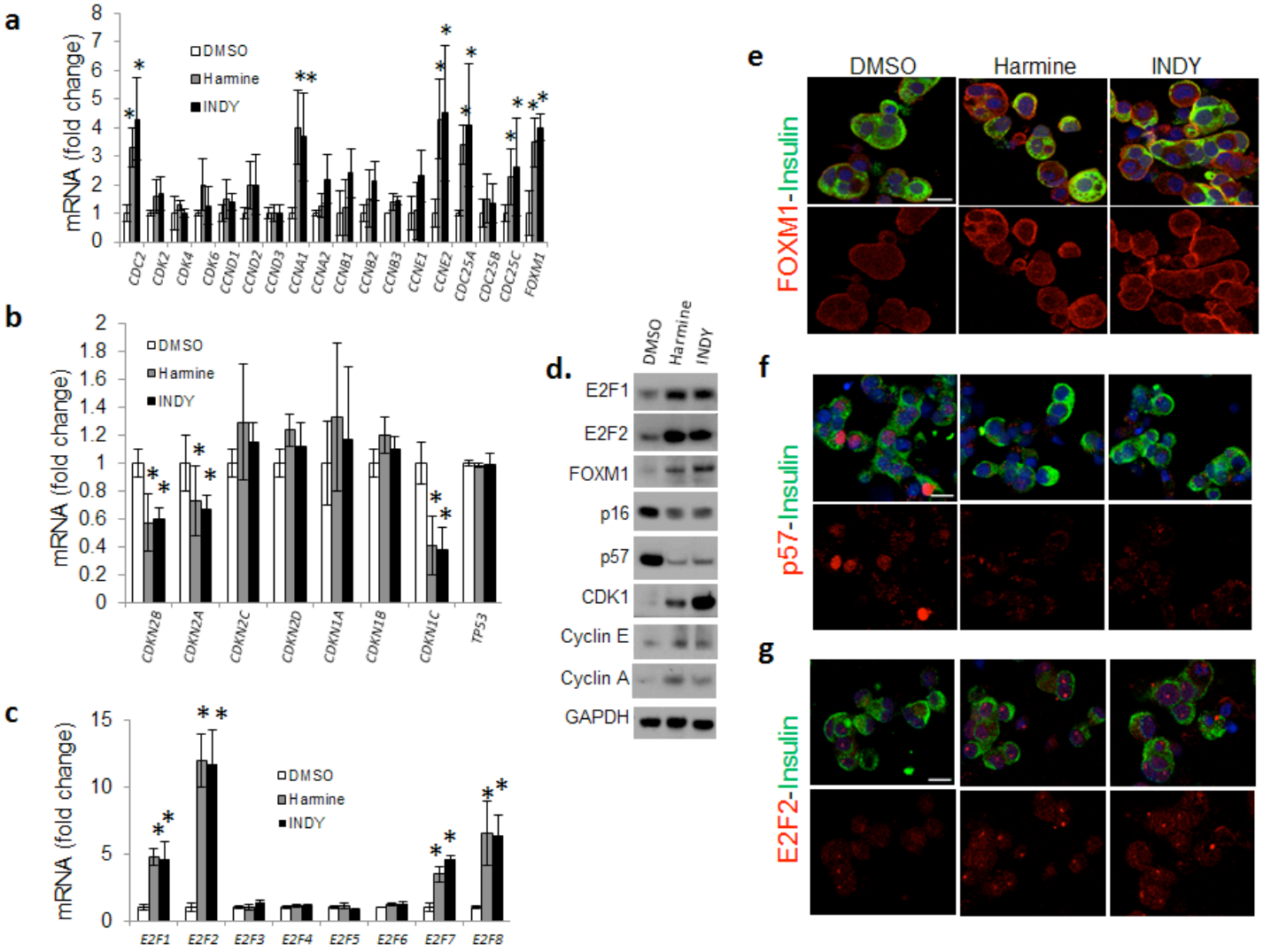
Supplementary Figure 6. Examples of immunolabeling and nuclear trafficking of endogenous NFATs1–4 in human islets. (**a**) Endogenous NFATs1–4 in human beta cells. "IgG/Blocking peptide" indicates that an NFAT1 blocking peptide was used as a negative control for NFAT1; for the other NFATs, non-specific IgG's were used as negative control. Representative of four different human islet preparations. (**b**) Harmine (10 μM) INDY and (15μM) treatment (three days) drives endogenous NFAT1 and NFAT3 translocation to the nucleus of human beta cells. Examples are indicated by red arrows. Representative of four different human islet preparations. In all relevant panels, the scale bars indicate 10 μm.



Supplementary Figure 7. Nuclear translocation of Ad.*NFATC1*-GFP and the ATP-binding pocket in DYRK1A. (**a**) A representative example of harmine (10 μM, 72 hr) and INDY (15 μM, 72 hr) treatment increasing the nuclear frequency of adenoviral *NFATC1*-GFP (encoding NFAT2-GFP) in human beta cells. The scale bar indicates 10 μm. (**b**) Quantification of nuclear frequency of Ad.*NFATC1*-GFP in four different preparations of human islets treated with harmine (10 μM, 72 hr) and INDY (15 μM, 72 hr). Error bars indicate s.e.m.; *indicates $P<0.05$ as determined by Wilcoxon Rank test. (**c** & **d**) Illustrations of harmine (**c**) and INDY (**d**) positioned in the ATP-binding pocket of DYRK1A, depicted as a gray surface. Harmine and INDY are shown in stick representation (green). The hydrogen bonds with residues Lys^{188} and Leu^{241} are shown as dashed yellow lines. The crystal structures correspond to PDB file 3anr (**c**) and 3anq (**d**)[20]. Figures prepared with The PyMOL Molecular Graphics System, Version 1.6.0.0 (Schrödinger, LLC).



Supplementary Figure 8. Low-level c-MYC expression is required for mouse, rat and beta cell proliferation mediated by harmine. (**a**) A c-MYC immunoblot of human islets treated with harmine (10 μM, 24 hr). (**b**) Densitometric quantification of c-MYC immunoblots from four human islet preparations. (**c**) Quantification of four different experiments in rat islets revealing the effects of the c-MYC inhibitor, 1RH (20 μM, 2 hr pretreatment) on harmine-induced beta cell proliferation. (**d**) Quantification of four different experiments in human islets revealing the effects of the c-MYC inhibitor, 1RH (20 μM, 2 hr pretreatment) on harmine-induced beta cell proliferation. (**e**) An example of DNA excision by Ad.Cre of *Myc* DNA in islets isolated from either wild-type ("WT") or $Myc^{loxP/loxP}$ mice. (**f**) c-Myc protein expression in the islets of $Myc^{loxP/loxP}$ mice following Ad.Cre treatment. (**g**) Quantification of Ki67 labeling of beta cells in islets of wild-type and $Myc^{loxP/loxP}$ mice transduced with Ad.Cre or Ad.LacZ and treated with harmine. (**h**) An example of Ki67 immunolabeling in beta cells in $Myc^{loxP/loxP}$ islets transduced with Ad.LacZ and treated with harmine. In all relevant panels, error bars indicate s.e.m.; *indicates $P<0.05$ and **indicates $P<0.01$ as determined by Wilcoxon Rank test. The scale bar in (**h**) indicates 10 μm.



Supplementary Figure 9. Harmine and INDY upregulate cell cycle activators and downregulate cell cycle inhibitors in human beta cells. (**a**–**c**) Real-time quantitative PCR of mRNAs encoding cell cycle activators and inhibitors in four different preparations of human islets treated with harmine (10 μM, 72 hr) or INDY (15 μM, 72 hr). Error bars indicate s.e.m.; *indicates $P<0.05$ as determined by Wilcoxon Rank test. (**d**) Representative immunoblots of key molecules upregulated in (**a**-**c**) from three different human islet preparations treated with harmine or INDY as in (**a**). (**e**) Representative images of immunocytochemistry from three different human islet donors for FOXM1, $p57^{KIP2}$, and E2F2 in response to harmine and INDY treatment as in (**a**). The scale bar indicates 10 μm.

Supplementary Table 1.

PCR Primers Used for RT-PCR.

Primer name	Sequence
CDKN2B forward	CGTTAAGTT TACGGCCAACG
CDKN2B reverse	GGTGAGAGTGGCAGGGTCT
CDKN2A forward	ATGGAGCCTTCGGCTGACT
CDKN2A reverse	GTAACTATTCGGTGCGTTGGG
CDKN2C forward	AAACTTGGAAATCCCGAGATTGC
CDKN2C reverse	CGAAACCAGTTCGGTCTTTCAA
CDKN2D forward	AGTCCAGTCCATGACGCAG
CDKN2D reverse	ATCAGGCACGTTGACATCAGC
CDKN1A forward	CGATGGAACTTCGACTTTGTCA
CDKN1A reverse	GCACAAGGGTACAAGACAGTG
CDKN1B forward	TAATTGGGGCTCCGGCTAACT
CDKN1B reverse	TGCAGGTCGCTTCCTTATTCC
CDKN1C forward	GCGGCGATCAAGAAGCTGT
CDKN1C reverse	GCTTGGCGAAGAAATCGGAGA
CDC2 forward	GGATGTGCTTATGCAGGATTCC
CDC2 reverse	CATGTACTGACCAGGAGGGATAG
CDK2 forward	GTACCTCCCCTGGATGAAGAT
CDK2 reverse	CGAAATCCGCTTGTTAGGGTC
CDK4 forward	TCAGCACAGTTCGTGAGGTG
CDK4 reverse	GTCCATCAGCCGGACAACAT
CDK6 forward	CCAGATGGCTCTAACCTCAGT
CDK6 reverse	AACTTCCACGAAAAAGAGGCTT
CCND1 Froward	CAATGACCCCGCACGATTTC
CCND1 Reverse	CATGGAGGGCGGATTGGAA
CCND2 Froward	TTTGCCATGTACCCACCGTC
CCND2 Reverse	AGGGCATCACAAGTGAGCG
CCND3 Froward	TACCCGCCATCCATGATCG

CCND3 Reverse	AGGCAGTCCACTTCAGTGC
CCNA1 Froward	GAGGTCCCGATGCTTGTCAG
CCNA1 Reverse	GTTAGCAGCCCTAGCACTGTC
CCNA2 Froward	GGATGGTAGTTTTGAGTCACCAC
CCNA2 Reverse	CACGAGGATAGCTCTCATACTGT
CCNB1 Froward	AATAAGGCGAAGATCAACATGGC
CCNB1 Reverse	TTTGTTACCAATGTCCCCAAGAG
CCNB2 Froward	TTGGCTGGTACAAGTCCACTC
CCNB2 Reverse	TGGGAACTGGTATAAGCATTGTC
CCNB3 Froward	ATGAAGGCAGTATGCAAGAAGG
CCNB3 Reverse	CATCCACACGAGGTGAGTTGT
CCNE1 Froward	ACTCAACGTGCAAGCCTCG
CCNE1 Reverse	GCTCAAGAAAGTGCTGATCCC
CCNE2 Froward	TCAAGACGAAGTAGCCGTTTAC
CCNE2 Reverse	TGACATCCTGGGTAGTTTTCCTC
C*DC25A* Forward	GTGAAGGCGCTATTTGGCG
CDC25A reverse	TGGTTGCTCATAATCACTGCC
CDC25B Forward	ACGCACCTATCCCTGTCTC
CDC25B reverse	CTGGAAGCGTCTGATGGCAA
CDC25C Forward	ATGACAATGGAAACTTGGTGGAC
CDC25C reverse	GGAGCGATATAGGCCACTTCTG
E2F1 Forward	CATCCCAGGAGGTCACTTCTG
E2F1 reverse	GACAACAGCGGTTCTTGCTC
E2F2 Forward	CGTCCCTGAGTTCCCAACC
E2F2 reverse	GCGAAGTGTCATACCGAGTCTT
E2F3 Forward	AGAAAGCGGTCATCAGTACCT
E2F3 reverse	TGGACTTCGTAGTGCAGCTCT
E2F4 Forward	CACCACCAAGTTCGTGTCCC
E2F4 reverse	GCGTACAGCTAGGGTGTCA
E2F5 Forward	ATGTCTTCTGACGTGTTTCCTC
E2F5 reverse	CGGGGTAGGAGAAAGCCTT
E2F6 Forward	TCCATGAACAGATCGTCATTGC

E2F6 reverse	TCCGTTGGTGCTCCTTATGTG
E2F7 Forward	TAGCTCGCTATCCAAGTTATCCC
E2F7 reverse	CAATGTCATAGATGCGTCTCCTT
E2F8 Forward	AAGTACGCCGAGCAGATTATG
E2F8 reverse	ATGTCTGGGTGTCCATTTGGG
FOXM1 Forward	ATACGTGGATTGAGGACCACT
FOXM1 reverse	TCCAATGTCAAGTAGCGGTTG
TPp53 Forward	GAGGTTGGCTCTGACTGTACC
TPp53 reverse	TCCGTCCCAGTAGATTACCAC
INS (Forward)	TCACACCTGGTGGAAGCTCTCTA
INS (Reverse)	ACAATGCCACGCTTCTGCAGGGAC
PDX1 (Forward	ACCAAAGCTCACGCGTGGAAA
PDX1 (Reverse)	TGATGTGTCTCTCGGTCAAGTT
NKX6-1 (Forward)	ACACGAGACCCACTTTTTCCG
NKX6-1 (Reverse)	TGCTGGACTTGTGCTTCTTCAAC
ACTB (Forward)	CATGTACGTTGCTATCCAGGC
ACTB (Reverse)	CTCCTTAATGTCACGCACGAT
Human *c-MYC* (Forward)	CCACACATCAGCACAACTACG
Human *c-MYC* (Reverse)	CAGCAGGATAGTCCTTCCGAG
MAFA (Forward)	GAGCGGCTACCAGCATCAC
MAFA (Reverse)	CTCTGGAGTTGGCACTTCTCG
Mouse *Myc-Flox* WT Forward	GCCCCTGAATTGCTAGGAAGACTG
Mouse *Myc*-Flox WT Reverse	CCGACCGGGTCCGAGTCCCTATT
Mouse *Myc-Flox* Deletion Forward	TCGCGCCCCTGAATTGCTAGGAA
Mouse *Myc-Flox* Deletion Reverse	TGCCCAGATAGGGAGCTGTGATACTT
Mouse *Actb* Forward	AGCCATGTACGTAGCCATCC
Mouse *Actb* Reverse	CTCTCAGCTGTGGTGGTGAA