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Supplemental information

An inducible CRISPR/Cas9 screen identifies DTX2 as a transcriptional regulator of human telomerase

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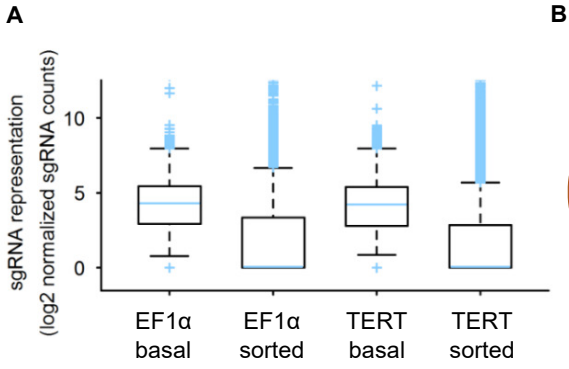
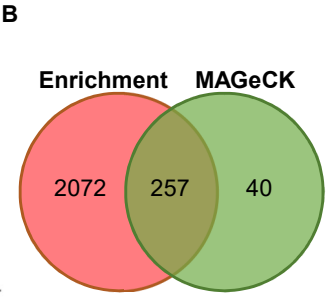
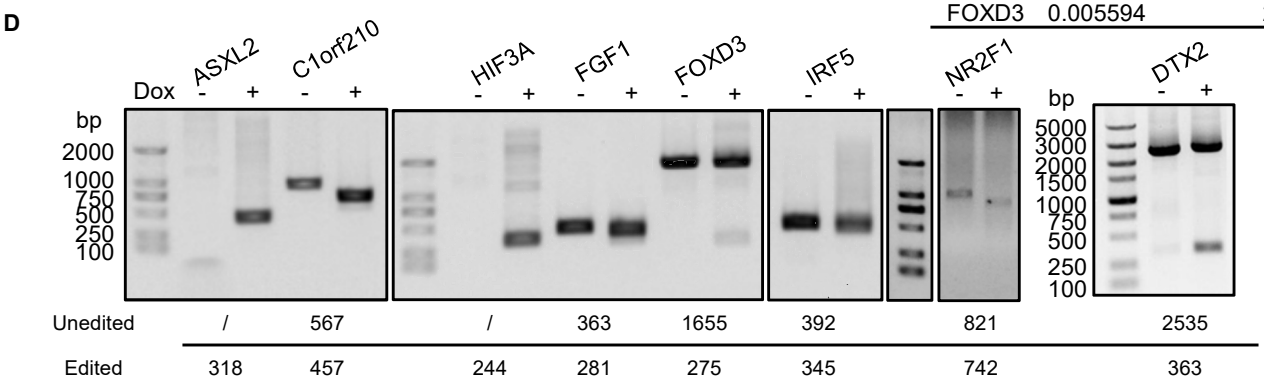
Fig. S1

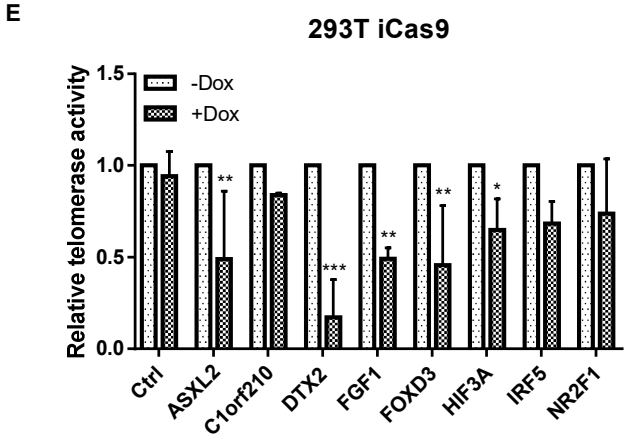


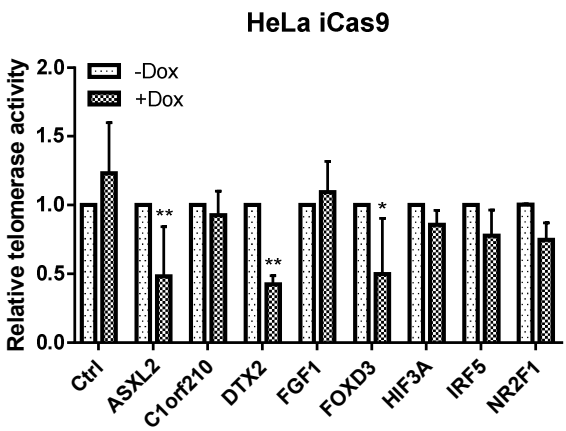


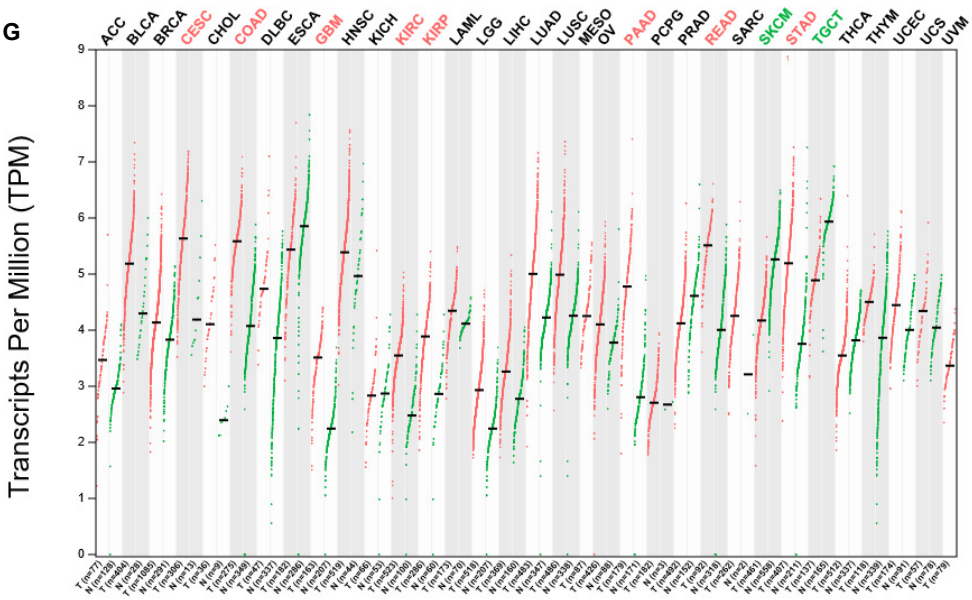
C

Gene ID	*P* value	Enriched sgRNA No.
DTX2	0.001495	4
ASXL2	0.003562	4
FGF1	0.000576	3
C1orf210	0.000605	3
HIF3A	0.004011	3
NR2F1	0.000228	2
IRF5	0.004893	2
FOXD3	0.005594	2









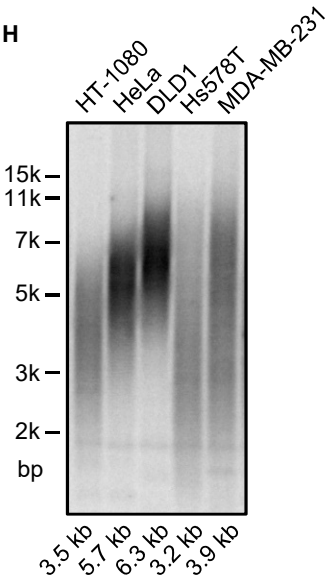


Fig. S1. An inducible CRISPR/Cas9 screen identifies positive regulators of *hTERT* transcription. Related to Figure 1.

(A) Box plot showed the distribution of sgRNA frequencies (both nuclear and unknown libraries) in the basal (-Dox) and sorted (+Dox and FACS sorting) samples. The horizontal blue line indicates median value.

(B) Enrichment analysis reveals 2,329 genes with targeting sgRNAs enriched in hTERT-EGFP samples. MAGeCK analysis identified 297 genes enriched (P<0.05) in hTERT-EGFP samples. The overlapping 257 genes were selected as candidates.

(C) The top eight candidate genes are listed with the number of enriched sgRNAs and *P* values from MAGeCK analysis.

(D) Inducible KO cell pools (used in Figures. 1D and 1E) were established using a dual-sgRNA strategy where vectors encoding inducible Cas9 were co-transfected into Hela or HEK293T cells with two sgRNAs targeting non-overlapping regions of the same gene. Cells were cultured -/+Dox (1 μg/mL) for 48 hours before genomic DNA extraction and PCR analysis of the regions spanning both sgRNA target sites. Agarose gel electrophoresis images of PCR reactions from each KO cell pool (-/+Dox) were shown, with the expected sizes of PCR products from un-edited and edited alleles listed below. "/" indicates regions too large for PCR amplification. The dual-sgRNA strategy may result in indels (at one or both sgRNA target sites) and/or deletions (precise or imprecise) of the intervening sequences between the two sgRNA target sites. The expected PCR product size given for edited alleles is based on precise deletions. In all the lines examined, a faster migrating band consistent with PCR products from precise (or close to precise) deletion could be detected, indicating efficient cleavage by both sgRNAs.

(E-F) The inducible KO cells from (D) were cultured -/+Dox (1 μg/mL) for 48 hours and then harvested for the qTRAP assay to determine relative telomerase activity in HEK293T iCas9 (D) and HeLa iCas9 (E) cells. Data were shown as mean ± SD (N=3). *P* values were calculated using Student's t test. *, P<0.05; **, P<0.01; ***, P<0.001.

(G) TCGA and GTEx RNA-seq data were analyzed using the GEPIA online tool to compare DTX2 mRNA levels in various cancer types and their normal tissue counterparts. DTX2 transcripts per million (TPM) were plotted in red for cancers and green for normal tissues. Cancer types are labeled in red if they have significantly higher DTX2 TPM values and green if significantly lower.

(H) Average telomere length of HT-1080, HeLa, DLD1, Hs578T, and MDA-MB-231 cell lines given beneath the blot was examined by the TRF assay and calculated using ImageQuant 5.2.

Fig. S2

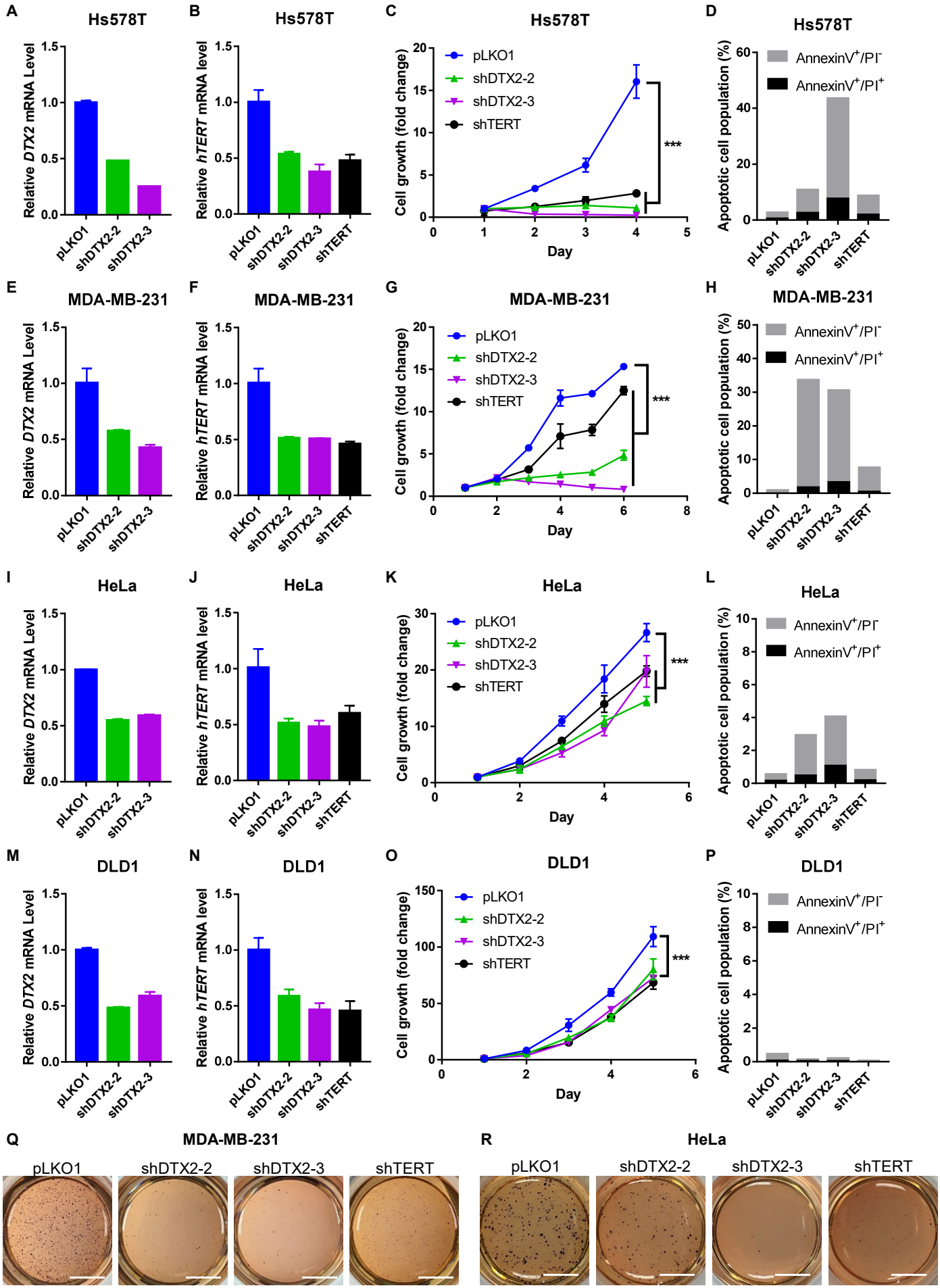

A
Hs578T
Relative DTX2 mRNA Level
pLKO1
shDTX2-2
shDTX2-3
B
Hs578T
Relative hTERT mRNA level
shTERT
C
Hs578T
Cell growth (fold change)
Day
***
D
Hs578T
Apoptotic cell population (%)
AnnexinV+/PI-
AnnexinV+/PI+
E
MDA-MB-231
F
MDA-MB-231
G
MDA-MB-231
H
MDA-MB-231
I
HeLa
J
HeLa
K
HeLa
L
HeLa
M
DLD1
N
DLD1
O
DLD1
P
DLD1
Q
MDA-MB-231
R
HeLa

Fig. S2. DTX2 is required for cell viability and anchorage-independent growth of cancer cells. Related to Figure 2.

DTX2 or *hTERT* shRNAs were stably expressed in telomerase-positive cancer cell lines Hs578T (A-D), MDA-MB-231 (E-H), HeLa (I-L), and DLD1 (M-P) by lentiviral transduction. Cells infected with pLKO1 empty vector viruses served as controls. Relative *DTX2* (A, E, I, M) and *hTERT* (B, F, J, N) mRNA levels were determined by RT-qPCR. At 72 hours after infection, cells were collected daily to monitor growth using the CCK8 assay (C, G, K, O). Data were shown as mean ± SD (N=4). ***, $P<0.001$. Apoptosis was assessed seven days after infection by Annexin V and PI staining, following by flow cytometry analysis (D, H, L, P). Annexin V^+/PI^- and Annexin V^+/PI^+ populations were defined respectively as early and late apoptotic cells.

(Q-R) MDA-MB-231 (Q) and HeLa (R) cells stably expressing *DTX2* or *hTERT* shRNAs were seeded in soft agar (top: 0.35%, bottom: 0.6%, 5,000 cells/well in 24-well plate) and cultured for 12 days. Live colonies were stained with MTT for 2 h and captured with camera (scale bar: 500 μm).

Fig. S3

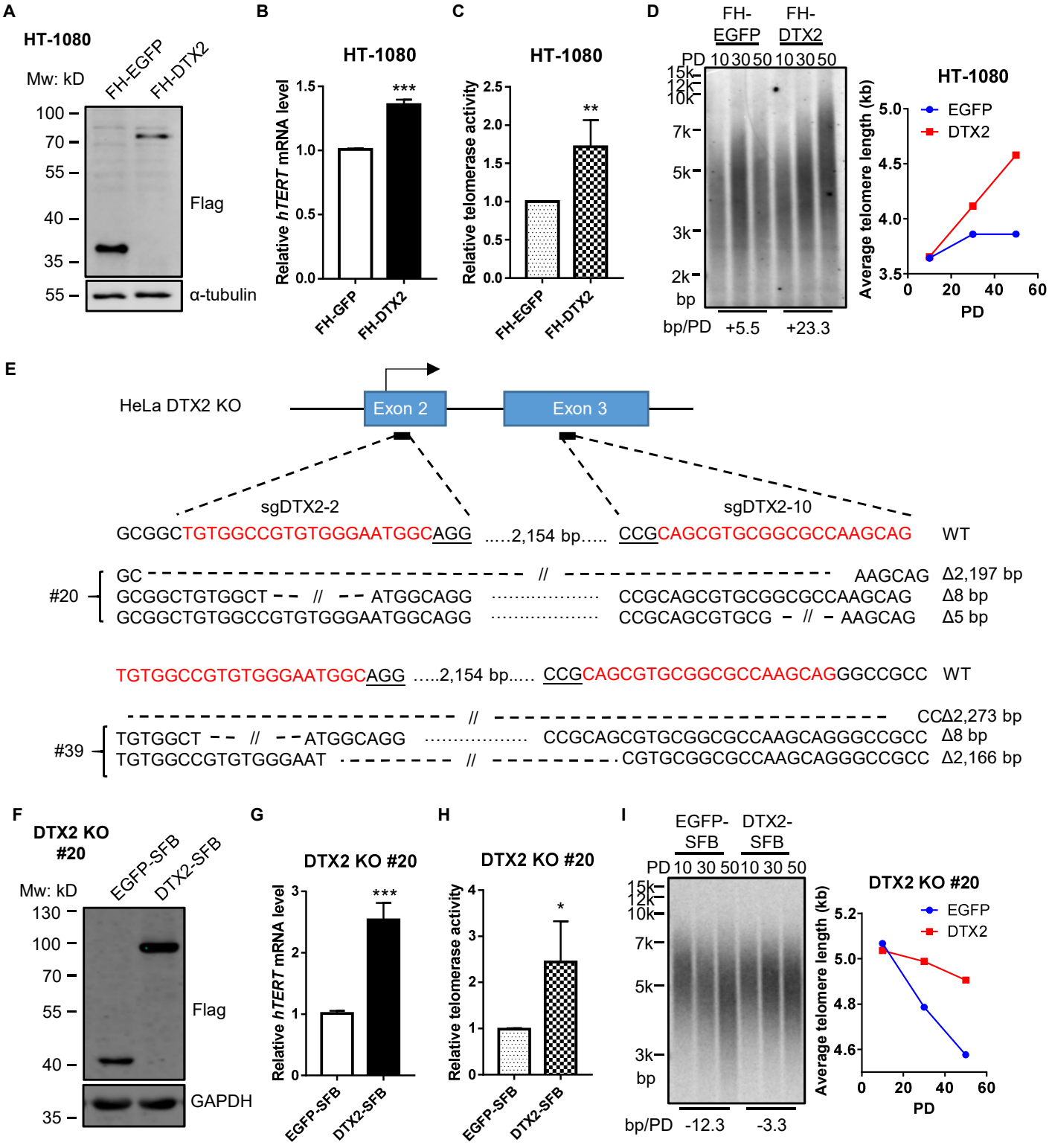

A
HT-1080
Mw: kD
FH-EGFP
FH-DTX2
100
70
55
40
35
55
Flag
α-tubulin
B
HT-1080
Relative hTERT mRNA level
1.5
1.0
0.5
0.0
***
FH-GFP
FH-DTX2
C
HT-1080
Relative telomerase activity
2.5
2.0
1.5
1.0
0.5
0.0
**
FH-EGFP
FH-DTX2
D
FH-EGFP
FH-DTX2
PD 10 30 50 10 30 50
15k
12k
10k
7k
5k
3k
2k
bp
bp/PD
+5.5
+23.3
HT-1080
Average telomere length (kb)
5.0
4.5
4.0
3.5
EGFP
DTX2
0
20
40
60
PD
E
HeLa DTX2 KO
Exon 2
Exon 3
sgDTX2-2
sgDTX2-10
GCGGCTGTGGCCGTGTGGGAATGGCAGG .....2,154 bp..... CCGCAGCGTGCGGCGCCAAGCAG WT
#20
GC // AAGCAG Δ2,197 bp
GCGGCTGTGGCT // ATGGCAGG ..... CCGCAGCGTGCGGCGCCAAGCAG Δ8 bp
GCGGCTGTGGCCGTGTGGGAATGGCAGG ..... CCGCAGCGTGCG // AAGCAG Δ5 bp
TGTGGCCGTGTGGGAATGGCAGG .....2,154 bp..... CCGCAGCGTGCGGCGCCAAGCAGGGCCGCC WT
#39
// CCΔ2,273 bp
TGTGGCT // ATGGCAGG ..... CCGCAGCGTGCGGCGCCAAGCAGGGCCGCC Δ8 bp
TGTGGCCGTGTGGGAAT // CGTGCGGCGCCAAGCAGGGCCGCC Δ2,166 bp
F
DTX2 KO #20
Mw: kD
EGFP-SFB
DTX2-SFB
130
100
70
55
40
35
Flag
GAPDH
G
DTX2 KO #20
Relative hTERT mRNA level
3
2
1
0
***
EGFP-SFB
DTX2-SFB
H
DTX2 KO #20
Relative telomerase activity
4
3
2
1
0
*
EGFP-SFB
DTX2-SFB
I
EGFP-SFB
DTX2-SFB
PD 10 30 50 10 30 50
15k
12k
10k
7k
5k
3k
bp
bp/PD
-12.3
-3.3
DTX2 KO #20
Average telomere length (kb)
5.2
5.0
4.8
4.6
EGFP
DTX2
0
20
40
60
PD

Fig. S3. DTX2 positively regulates *hTERT* transcription. Related to Figure 2.

(A-D) Flag-HA-tagged DTX2 (FH-DTX2) or EGFP (FH-EGFP) was expressed in HT-1080 cells. The expression of FH-DTX2 and FH-EGFP was examined by western blotting with an anti-Flag antibody (A). Relative *hTERT* mRNA level (B) and telomerase activity (C) were determined by RT-qPCR and qTRAP respectively. Data were shown as mean ± SD (N=3). *P* values were calculated using Student's t test. **, $P<0.01$; ***, $P<0.001$. Average telomere length was examined by the TRF assay and calculated using ImageQuant 5.2 (D). Average telomere length change is given beneath the TRF blot.

(E) To generate DTX2 KO lines, Cas9 and two sgRNAs (sgDTX2-2 and sgDTX2-10) targeting different DTX2 exons were transiently transfected into HeLa cells. The sgRNA (in red) and PAM (underlined) sequences are shown. Two DTX2 KO lines (#20 and #39) were selected and their genomic regions encompassing the two sgRNA target sites were amplified. The amplicons were ligated into pEASY-Blunt Zero vectors for Sanger sequencing and the results are presented here.

(F-I) SFB-tagged EGFP or DTX2 was expressed in DTX2 KO line #20. The cells were examined for protein expression (F), relative *hTERT* mRNA level (G), telomerase activity (H), and average telomere length (I). Data were shown as mean ± SD (N=3). *P* values were calculated using Student's t test. *, $P<0.05$; ***, $P<0.001$. Average telomere length change is given beneath the TRF blot.

Fig. S4

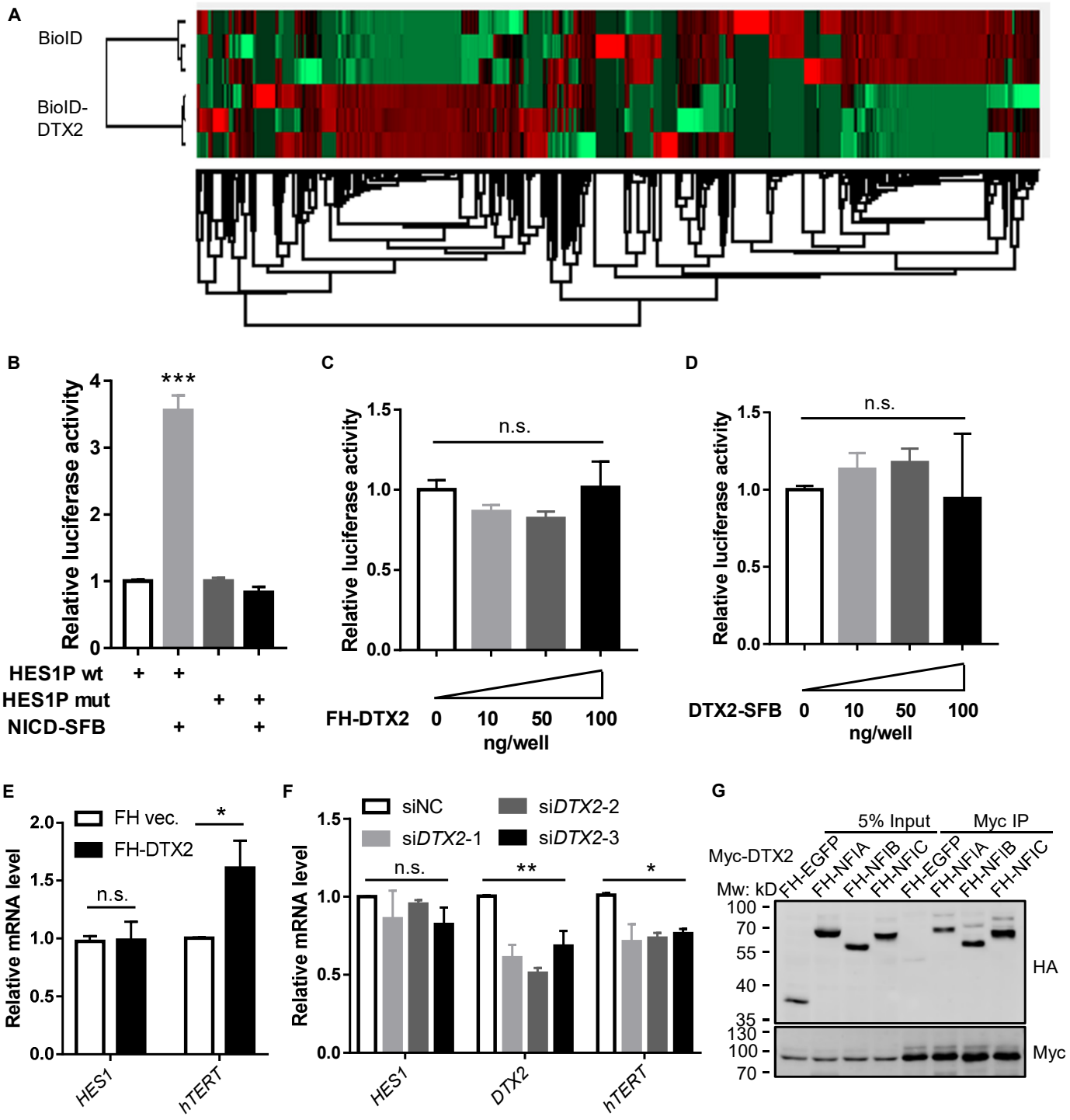

A
BioID
BioID-DTX2
B
Relative luciferase activity
***
HES1P wt
HES1P mut
NICD-SFB
C
Relative luciferase activity
n.s.
FH-DTX2
0
10
50
100
ng/well
D
Relative luciferase activity
n.s.
DTX2-SFB
0
10
50
100
ng/well
E
Relative mRNA level
FH vec.
FH-DTX2
n.s.
*
HES1
hTERT
F
Relative mRNA level
siNC
siDTX2-1
siDTX2-2
siDTX2-3
n.s.
**
*
HES1
DTX2
hTERT
G
5% Input
Myc IP
Myc-DTX2
Mw: kD
FH-EGFP
FH-NFIA
FH-NFIB
FH-NFIC
100
70
55
40
35
130
100
70
HA
Myc

Fig. S4. BioID-mediated proximity-labeling identifies DTX2-associating partners. Related to Figure 5.

(A) The heatmap was drawn using Perseus based on fraction of total (FOT) values for proteins identified from each group. Red and green indicate high and low FOT values respectively.

(B) Vectors encoding the SFB-tagged Notch intracellular domain (NICD-SFB) (100 ng) were co-transfected into HEK293T cells with a luciferase reporter driven by wild-type (HES1P-wt) or mutant (HES1P-mut) HES1 promoter (20 ng) and pRL-SV40 (5 ng) as internal control. Relative luciferase activities were determined 24 hours later. Data were shown as mean ± SD (N=6). *P* values were calculated using Student's t test. ***, $P<0.001$.

(C-D) The luciferase reporter driven by the HES1-WT promoter (20 ng) and internal control pRL-SV40 (5 ng) were co-transfected into HEK293T cells with increasing amount of plasmids encoding FH-DTX2 (C) or DTX2-SFB (D). Total amount of DNA was made constant with the addition of empty vector (up to 100 ng). Relative luciferase activities in the cells were determined 24 hours later. Data were shown as mean ± SD (N=6). *P* values were calculated using Student's t test. n.s., not significant.

(E-F) HeLa cells stably expressing FH-DTX2 (E) or transfected with siRNAs targeting DTX2 (F) were harvested to assess the relative mRNA levels of *HES1, DTX2,* and *hTERT*. Vector alone and non-targeting siRNAs served as controls. Data were shown as mean ± SD (N=3). *P* values were calculated using Student's t test. *, $P<0.05$; **, $P<0.01$; n.s., not significant.

(G) HEK293T cells transiently co-expressing myc-tagged DTX2 (myc-DTX2) and FH-tagged NF-I family proteins were harvested for co-immunoprecipitation (co-IP) assays with an anti-myc antibody. The immunoprecipitates were blotted as indicated.

Fig. S5

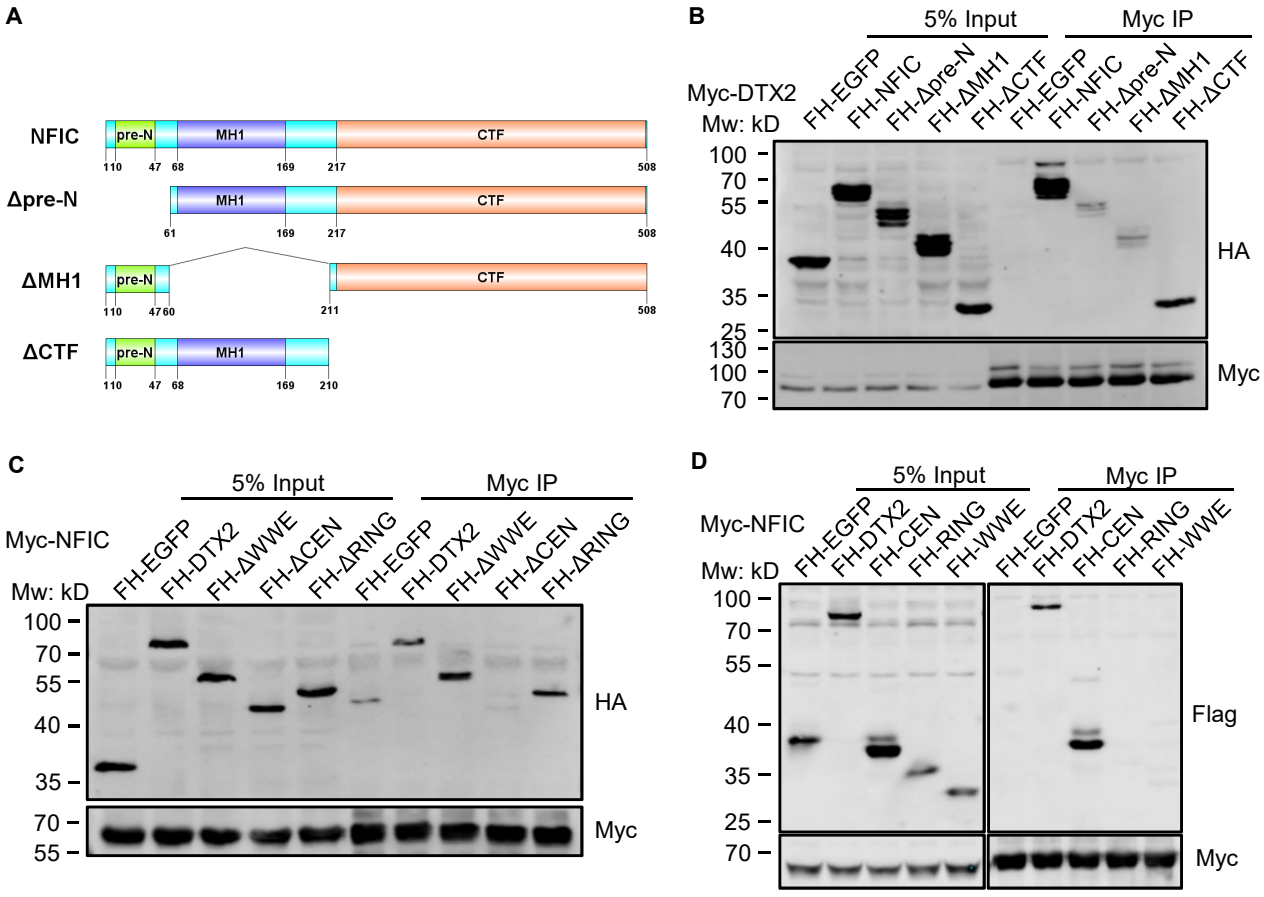

A
NFIC
pre-N
MH1
CTF
110
47
68
169
217
508
Δpre-N
61
ΔMH1
60
211
ΔCTF
210
B
5% Input
Myc IP
Myc-DTX2
Mw: kD
FH-EGFP
FH-NFIC
FH-Δpre-N
FH-ΔMH1
FH-ΔCTF
100
70
55
40
35
25
130
HA
Myc
C
Myc-NFIC
FH-DTX2
FH-ΔWWE
FH-ΔCEN
FH-ΔRING
D
FH-CEN
FH-RING
FH-WWE
Flag

Fig. S5. The central region of DTX2 interacts with the N-terminus of NFIC. Related to Figure 5.

(A) Schematic diagram of full-length NFIC and its truncation mutants. Pre-N, the highly conserved extreme N-terminal end of NFIC. MH1, Mad Homology 1 domain. CTF, CTF/NFI family transcription modulation region.

(B) HEK293T cells transiently co-expressing myc-tagged DTX2 (myc-DTX2) with FH-tagged full-length NFIC or its truncation mutants were harvested for co-immunoprecipitation (co-IP) assays with an anti-myc antibody. The immunoprecipitates were blotted as indicated.

(C) HEK293T cells transiently co-expressing myc-tagged NFIC (myc-NFIC) with FH-tagged full-length DTX2 or its truncation mutants were harvested for co-immunoprecipitation (co-IP) assays with an anti-myc antibody. The immunoprecipitates were blotted as indicated.

(D) HEK293T cells transiently co-expressing myc-tagged NFIC (myc-NFIC) with FH-tagged full-length DTX2 and its truncation mutants were harvested for co-immunoprecipitation (co-IP) assays with an anti-myc antibody. The immunoprecipitates were blotted as indicated.

Fig. S6

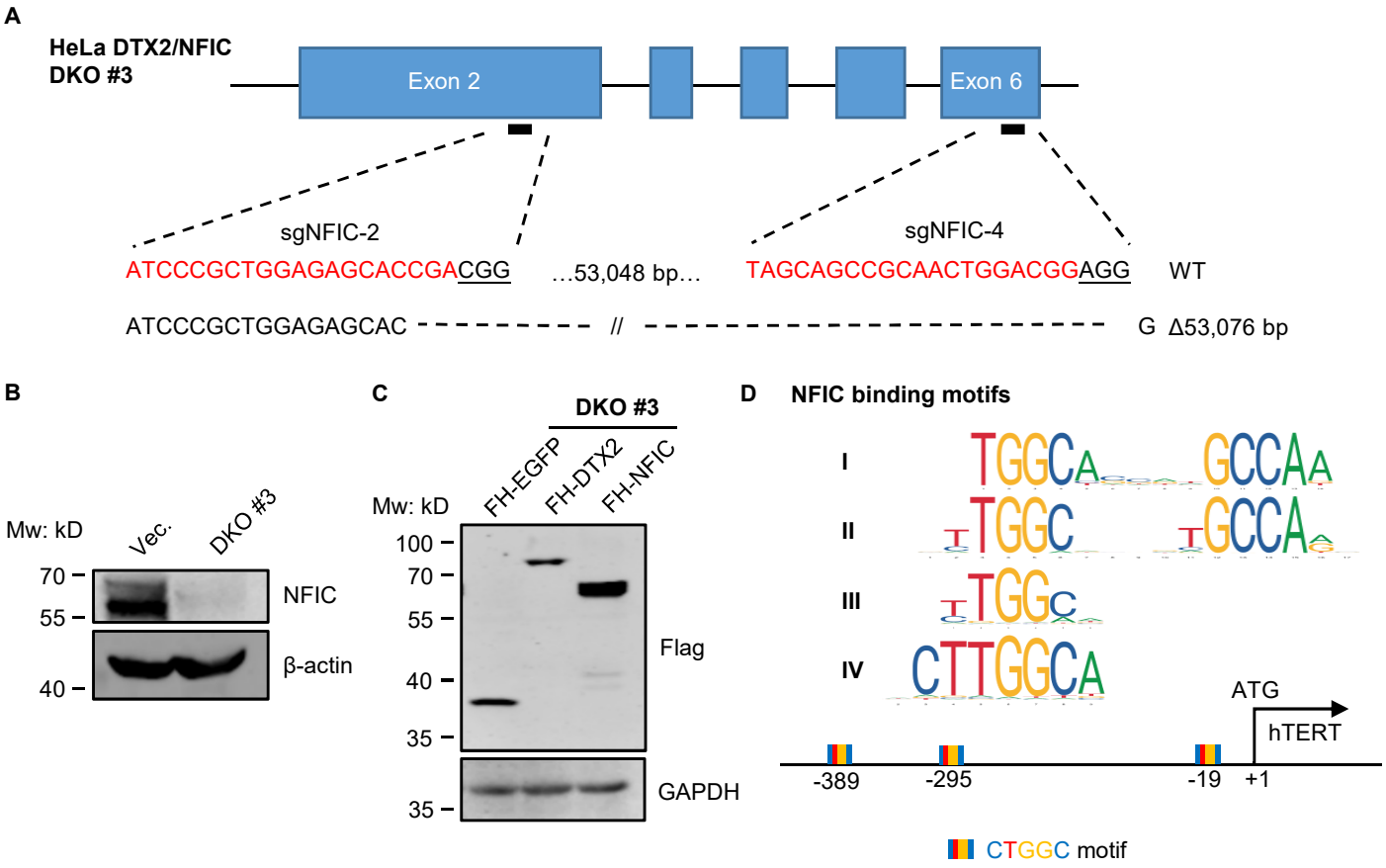

A
HeLa DTX2/NFIC
DKO #3
Exon 2
Exon 6
sgNFIC-2
sgNFIC-4
ATCCCGCTGGAGAGCACCGACGG
…53,048 bp…
TAGCAGCCGCAACTGGACGGAGG
WT
ATCCCGCTGGAGAGCAC
//
G Δ53,076 bp
B
Mw: kD
Vec.
DKO #3
70
55
40
NFIC
β-actin
C
DKO #3
FH-EGFP
FH-DTX2
FH-NFIC
Mw: kD
100
70
55
40
35
35
Flag
GAPDH
D
NFIC binding motifs
I
II
III
IV
ATG
hTERT
-389
-295
-19
+1
CTGGC motif

Fig. S6. Generation of DTX2/NFIC DKO cells and schematic of NFIC-binding motifs. Related to Figure 6.

(A) To generate DTX2/NFIC DKO cell lines, vectors encoding Cas9 and two sgRNAs (sgNFIC-2 and sgNFIC-4) targeting different NFIC exons were transiently transfected into DTX2 KO cell (#20). The sgRNA (red) and PAM (underlined) sequences were shown. DKO line #3 was selected with its genomic target regions amplified and ligated into pEASY-T Blunt vectors for Sanger sequencing, which indicates successful homozygous deletion of the intervening sequences between the two sgRNA target sites.

(B) DKO line #3 was examined by western blot with an anti-NFIC antibody. DTX2 KO line transfected with empty px458 vector served as control.

(C) DTX2/NFIC DKO cells stably expressing FH-tagged EGFP, DTX2, or NFIC were examined by western blot using the indicated antibodies.

(D) Several DNA-binding motifs of NFIC were identified in SELEX and ChIP-seq data from the JASPAR database (top). Three CTGGC motifs are found within the hTERT core promoter, located around -19 bp, -295 bp, and -389 bp relative to the translation start site (bottom).

Fig. S7

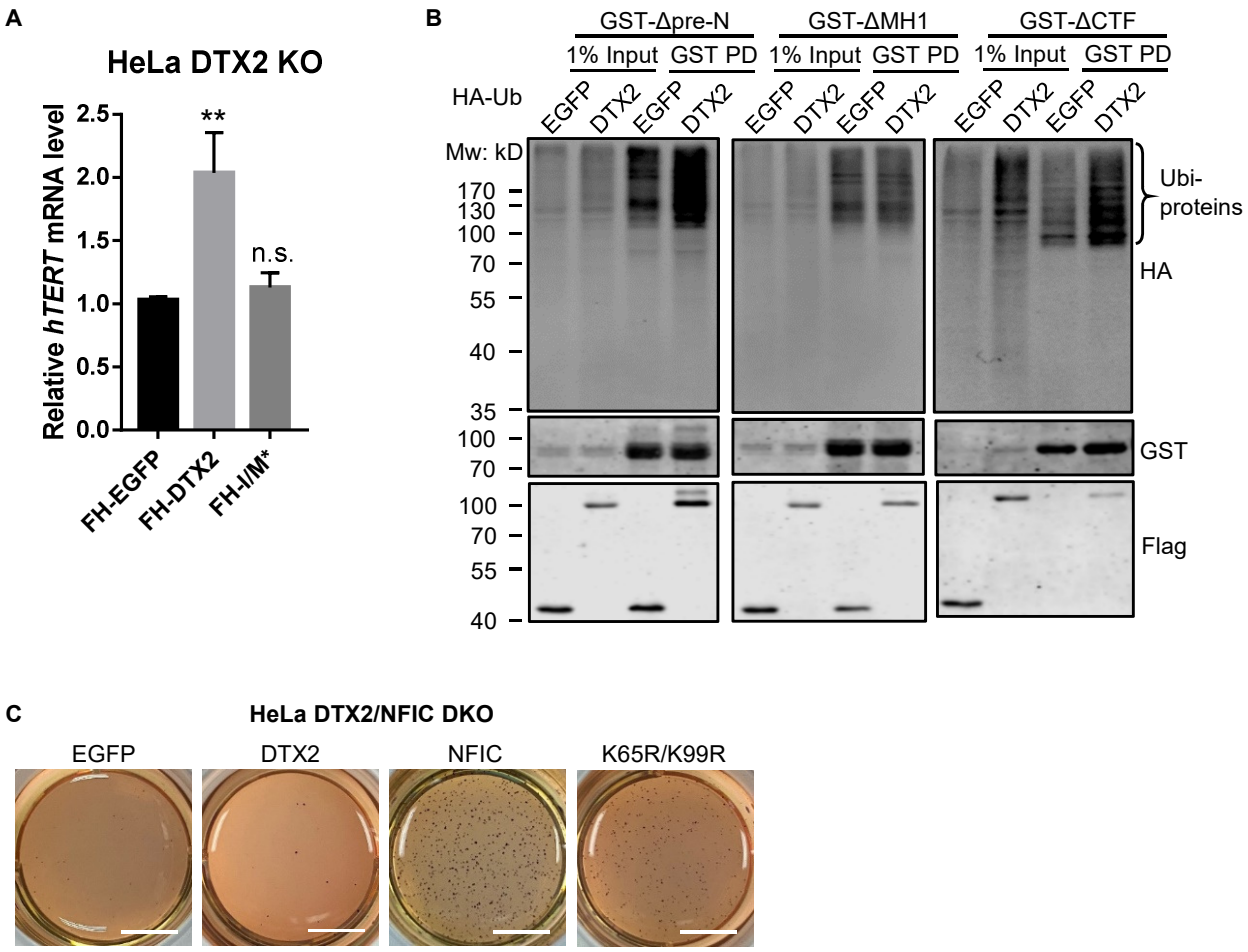

A
HeLa DTX2 KO
Relative hTERT mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
**
n.s.
FH-EGFP
FH-DTX2
FH-I/M*
B
GST-Δpre-N
GST-ΔMH1
GST-ΔCTF
1% Input
GST PD
HA-Ub
EGFP
DTX2
Mw: kD
170
130
100
70
55
40
35
Ubi-proteins
HA
GST
Flag
C
HeLa DTX2/NFIC DKO
EGFP
DTX2
NFIC
K65R/K99R

Fig. S7. DTX2-mediated ubiquitination of NFIC contributes to tumorigenesis. Related to Figure 7.

(A) DTX2 KO HeLa cells with re-expression of WT DTX2 or DTX2 I/M* (loss of enzymatic activity) were generated. Relative *hTERT* mRNA levels were determined by RT-qPCR. Data were shown as mean ± SD (N=3). *P* values were calculated using Student's t test. **, $P<0.01$; n.s., not significant.

(B) HA-Ub was transiently co-expressed in HEK293T cells with DTX2-SFB as well as GST-tagged full-length or truncation mutants of NFIC as indicated. EGFP-SFB was used as a control. The cells were harvested for GST pulldown (PD) and western blot as shown.

(C) DTX2/NFIC DKO HeLa cells stably expressing DTX2, WT NFIC or mutants (EGFP expressing group served as control) were seeded in soft agar (top: 0.35%, bottom: 0.6%, 10,000 cells/well in 24-well plate) and cultured for 16 days. Live colonies were stained with MTT for 2 h and captured with camera (scale bar: 500 μm).