Supplementary Material:
Targeting USP13 mediated drug tolerance increases the efficacy of EGFR inhibition of mutant EGFR in Non-Small Cell Lung Cancer

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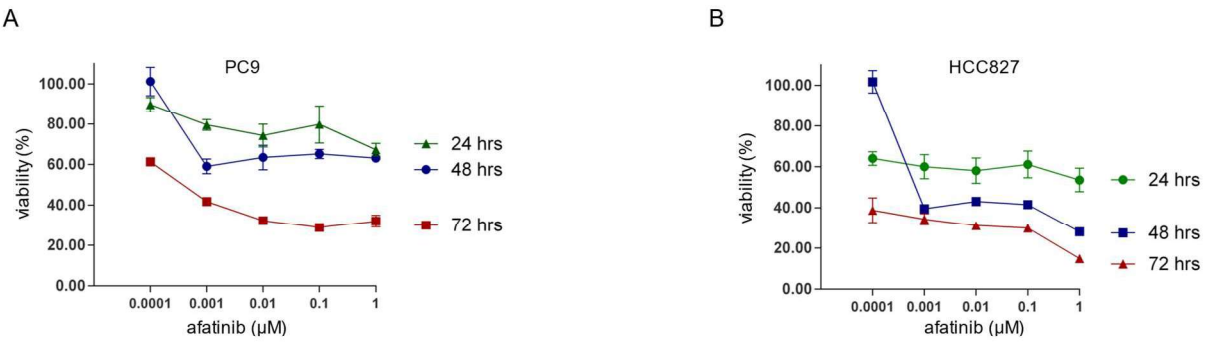

Figure S1. Titration of afatinib in PC9 and HCC827 cells.

PC9 (A) and HCC827 (B) cells were treated with afatinib at the indicated doses for 24, 48 and 72 h. Results were normalized to the DMSO control of each time-point. Dots represent mean viability (%) ± SD.

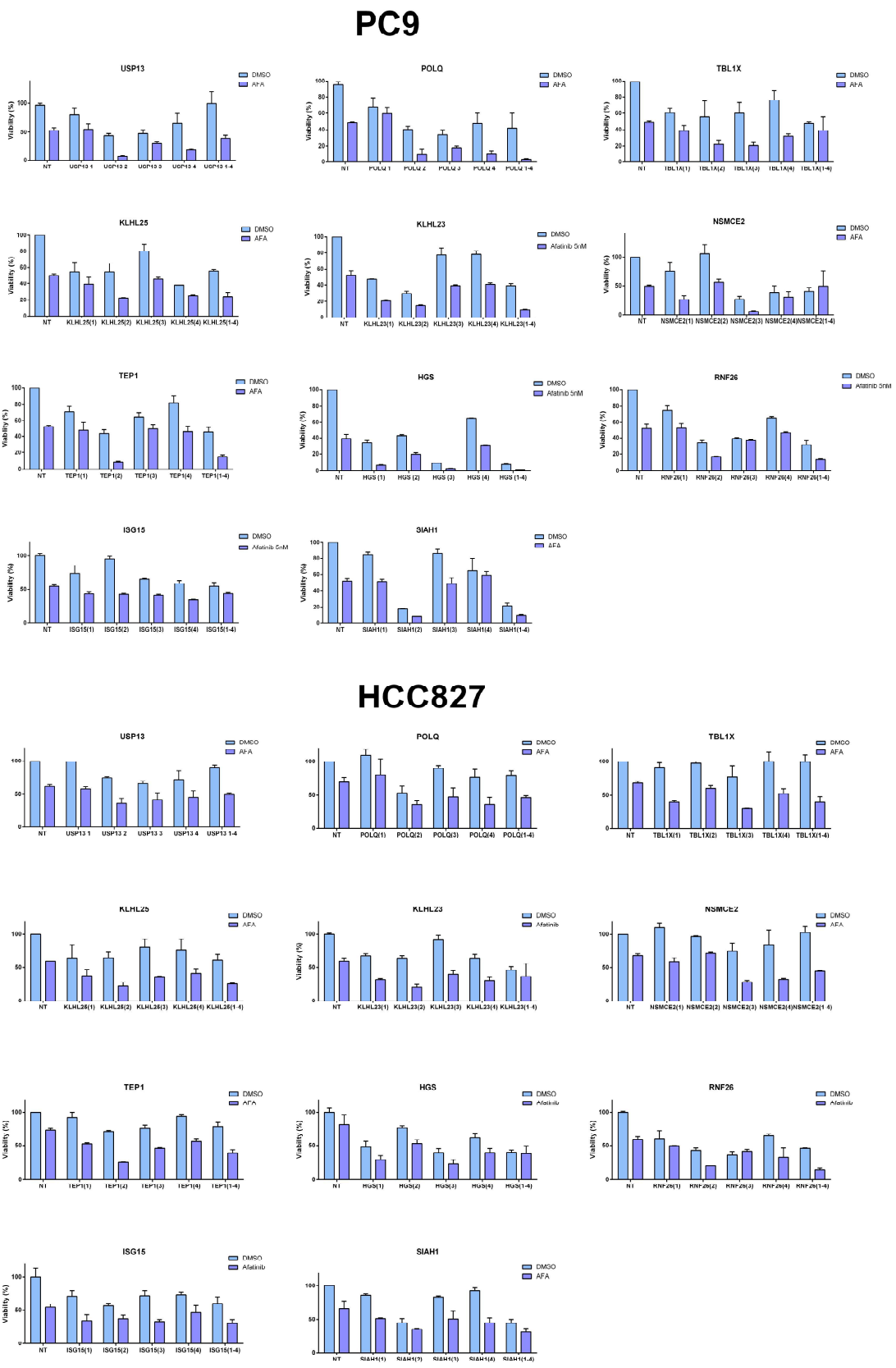

Figure S2. Validation of the individual siRNA pools in PC9 and HCC827 cells.

PC9 (top) and HCC827 (bottom) cells were reverse-transfected with the indicated siRNAs and treated with afatinib or vehicle (DMSO) 48 h post-transfection. Cell viability was measured 96 h post-transfection using the CellTiter-Glo assay. Results were normalized to the non-targeting (NT) siRNA DMSO control. Bars represent mean viability (%) ± SEM. Experiments were performed in duplicate.

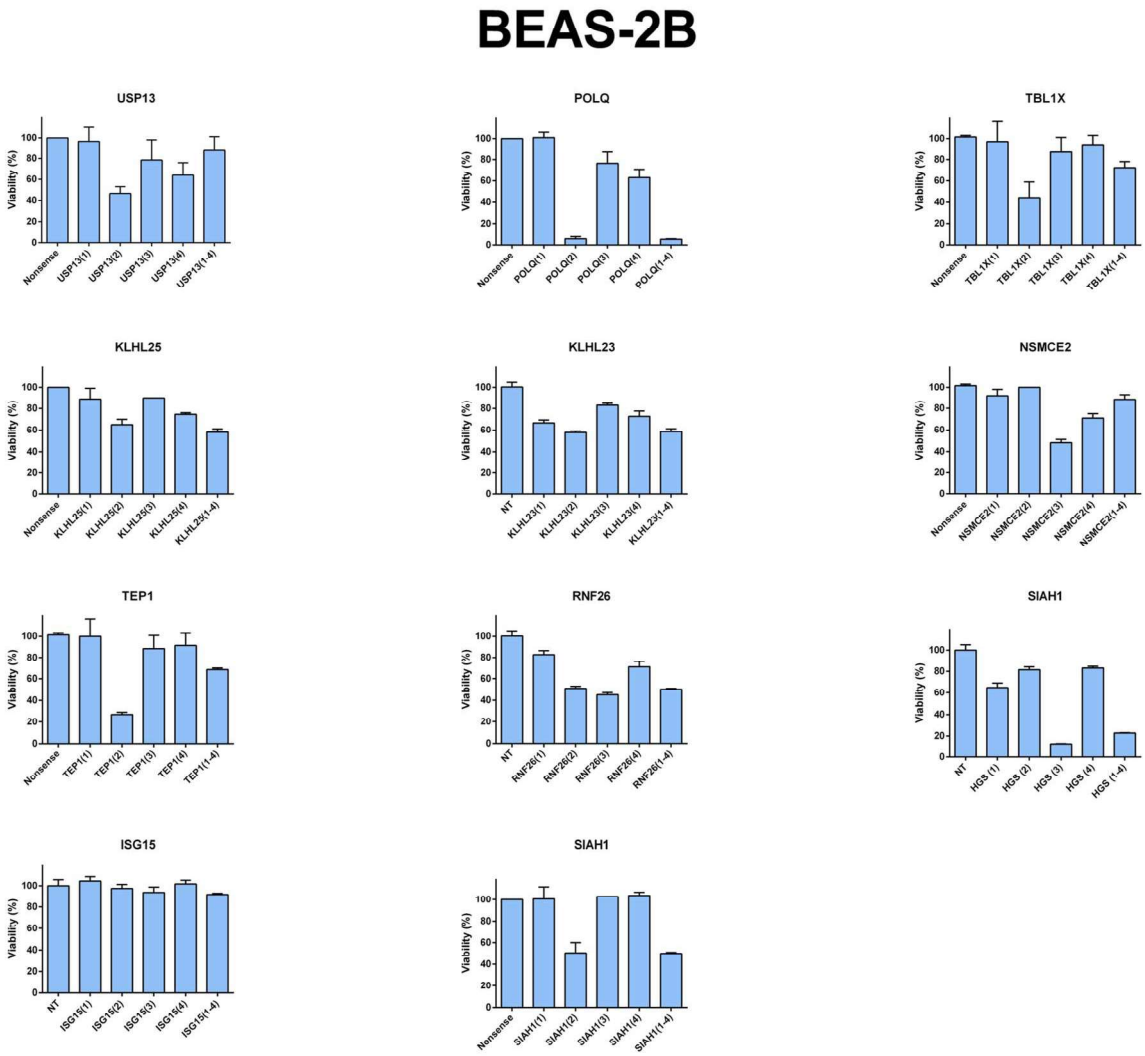

Figure S3. Validation of the individual siRNA pools in BEAS-2B cells.

BEAS-2B cells were reverse-transfected with the indicated siRNAs. Cell viability was measured 96 h post transfection using the CellTiter-Glo assay. Results were normalized to the non-targeting (NT) siRNA control. Bars represent mean viability (%) ± SEM. Experiments were performed in duplicate.

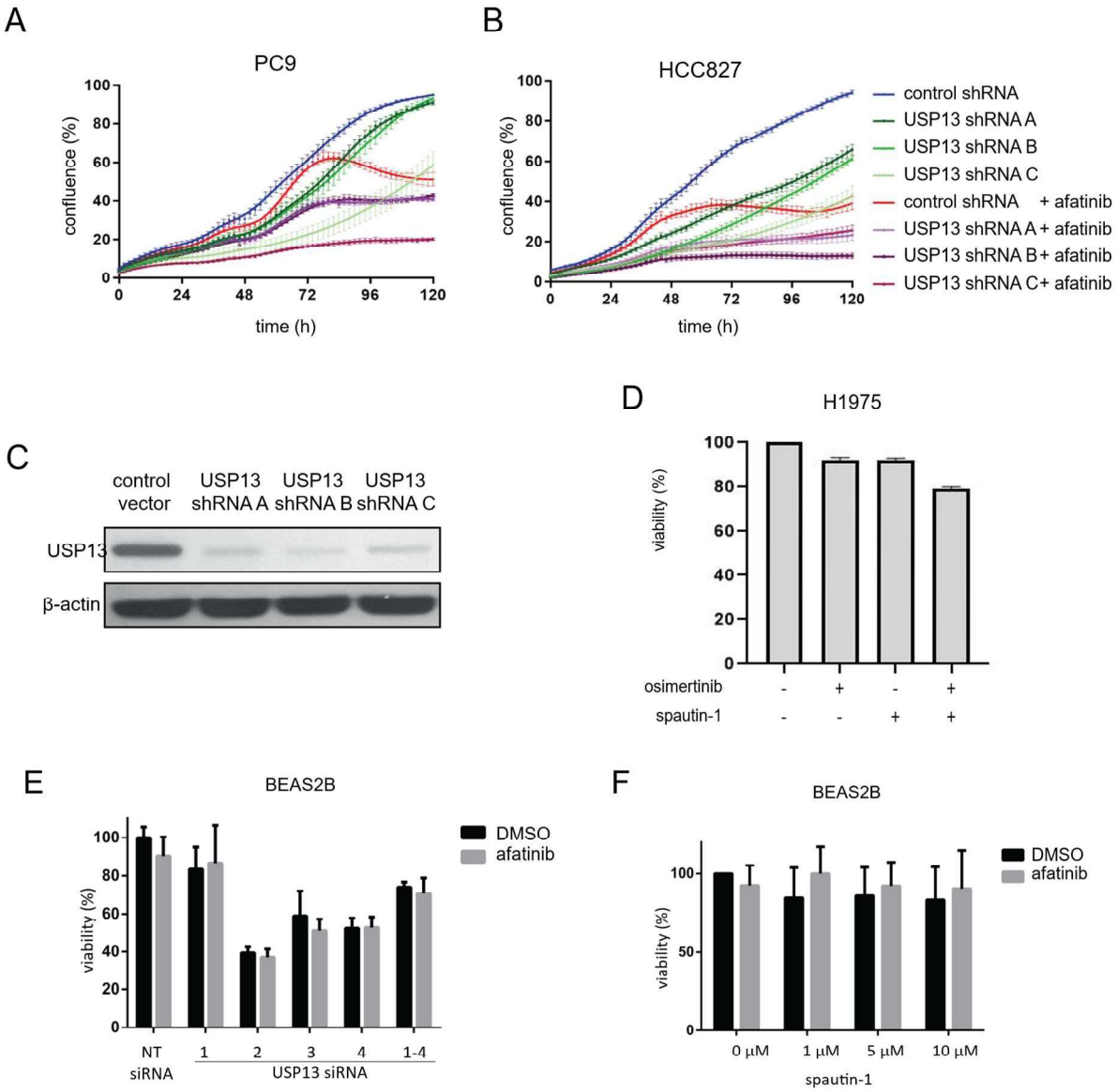

Figure S4. Cellular effects of USP13 knock-down and inhibition

(A) Confluence analysis of PC9 and HCC827 treated with afatinib, spautin-1 or their combination for 120 h. Lines represent the lowess curve through actual data points; the SEM is shown by bars per data point. Confluence was determined every hour. The graph is representative of three independent experiments. (B) As in (A) but using cells stably transduced with pLKO.1 shRNA vectors empty (control) or expressing USP13 shRNAs A-C. (C) USP13 knock-down by shRNAs in PC9 cells. PC9 cells were stably normalized to the NT siRNA DMSO control. Bars represent mean viability (%) ± SEM. (D) Cell viability analysis of H1975 treated with osimertinib, spautin-1 and the combination. Bars represent mean viability (%) ± SEM. (E) Cell viability analysis of BEAS-2B treated with afatinib, USP13 siRNA, or their combination for 72 hrs. Bars represent mean ± SEM. (F) as in (E) but with varying concentrations of spautin-1 (as indicated) in combination with afatinib.

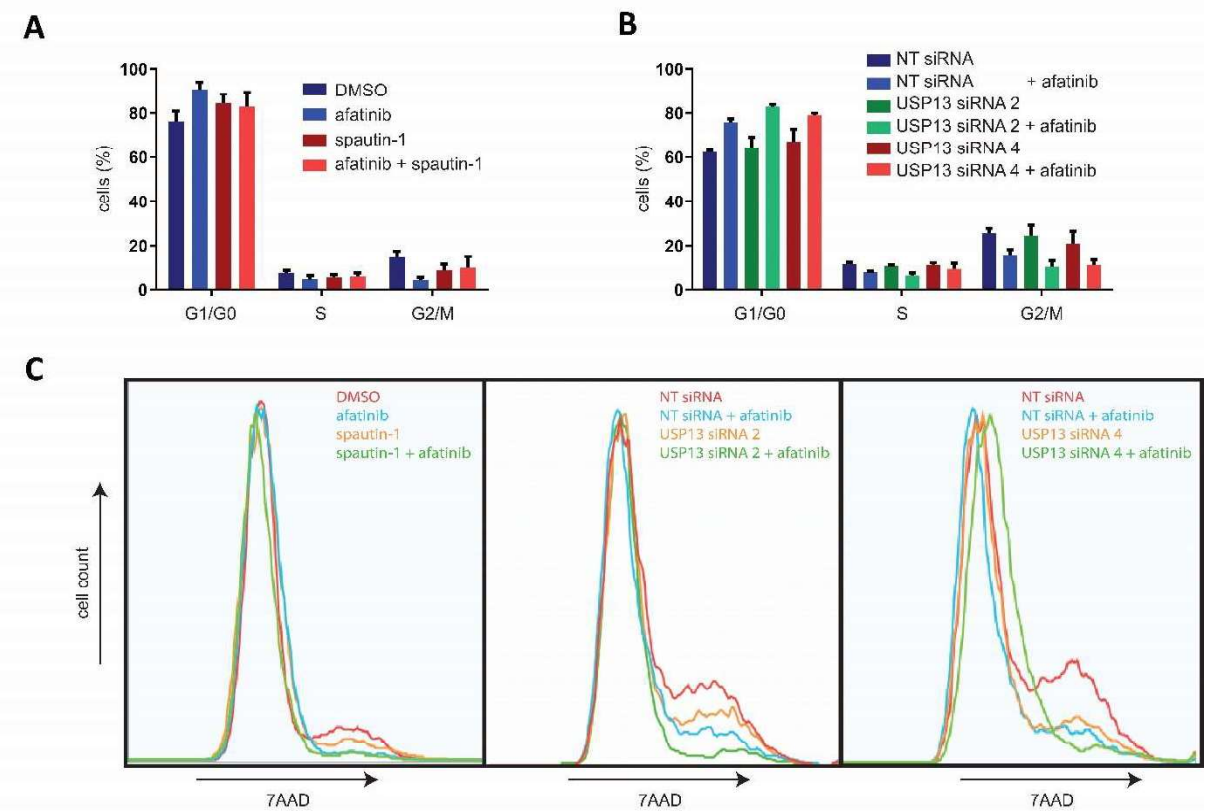

Figure S5. Cell cycle analysis

(A) Cell cycle analysis of PC9 cells treated with afatinib, spautin-1 or their combination, determined 48 h post-treatment by flow cytometry analysis of 7-AAD stained cells. Bars represent the mean ± SEM. (B) As in (A), but using cells reverse-transfected with indicated siRNAs and treated with afatinib or vehicle (DMSO) 48 h post-transfection. (C) Representative graphs of the 7-AAD staining from (A-B).

Supplementary Materials and Methods

Primers

EGFR forward primer (FW) 1 (5'-GGTGACTGTTTGGGAGTTGA-3'), EGFR reverse primers (RV) 1 (5'-TCAACTCACGGAACTTTGGG-3'), EGFR FW2 (5'-GGCACTTTTGAAGATCATTTTCTC-3'), EGFR RV2 (5'-CTGTGTTGAGGGCAATGAG-3'), EGFR FW3 (5'-GGCAGGAGTCATGGGAGAA-3'), EGFR RV3 (5'-GCGATGGACGGGATCTTAG-3'), USP13 FW1 (5'-GCGAAATCAGGCTATTCAGG-3'), USP13 RV1 (5'-TTGTAAAATCACCCATCTTCCTTCC-3'), USP13 FW2 (5'-TCTCGCTTTGCTTCATTCCCT-3'), USP13 RV2 (5'-TGGTTCATCAGGCGATCTTTTG-3'), SDHA FW1 (5'-TGGGAACAAGAGGGCATCTG-3'), SDHA RV1 (5'-CCACCACTGCATCAAATTCATG-3'), TBP FW1 (5'-CACGAACCACGGCACTGATT-3'), TBP RV1 (5'-TTTTCTTGCTGCCAGTCTGGAC-3').

Antibodies

EGFR (also for Confocal imaging): Cat#AMAb90816 (Merck); EGFR (also for IP): Cat#sc-03-G (Santa Cruz); phospho-EGFR (Tyr1068): Cat##2234 (Cell Signaling); p44/42 MAPK (Erk1/2): Cat#4695 (Cell Signaling); phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204): Cat#9101 (Cell Signaling); c-Cbl: Cat#HPA027956 (Merck); USP13: Cat#HPA004827 (Merck); Akt: Cat#9272 (Cell Signaling); phospho-Akt (Ser473): Cat#9271 (Cell Signaling); α-tubulin: Cat#T9026 (Merck); β-actin: Cat#A1978 (Merck); FLAG: Cat#2368 (Cell Signaling); HA: Cat#2367 (Cell Signaling), anti-mouse Alexa Fluor 647 (AB_2492288; red) and anti-rabbit Alexa Fluor 488 (AB_2340846; green) (Jackson ImmunoResearch Europe).

DNA plasmids and mRNA silencing constructs

The following plasmids were used: p3XFLAG-CMV-14 (Merck); pcDNA4/myc-His B (ThemoFisher); pEF-FLAG-USP13 (Chen et al., 2011); pCMV6-XL-USP13 (OriGene); HA-c-Cbl (kindly provided by Prof. Kwang Y. Lee (Chonnam National University, South Korea)); p3XFLAG-CMV-14 Cbl-b (cloned from Cbl-b cDNA kindly provided by Prof. Kwang Y. Lee); pcDNA4-EGFR-myc-His B (kindly provided by Dr.

Yi-Rong Chen (National Health Research Institutes, Taiwan); pcDNA4-EGFR-(L858R)-myc-His B (site-directed mutagenesis); pcDNA4-EGFR-(ΔE746-750)-myc-His B (site-directed mutagenesis).

The siRNA constructs used were ON-TARGETplus Human USP13 siRNAs (Dharmacon); siRNA1 (J-006064-05); siRNA2 (J-006064-06); siRNA3 (J-006064-07); siRNA4 (J-006064-08); and the ON-TARGETplus Non-targeting Control Pool (D-001810-10-05) as control siRNA. The shRNA constructs were: MISSION® pLKO.1-puro Non-Target shRNA Control Plasmid (Merck; SHC016-1EA) and the USP13 MISSION shRNA plasmids (Merck): shRNA A (TRCN0000007250); shRNA B (TRCN0000007251); and shRNA C (TRCN0000007252).