

Expanded View Figures

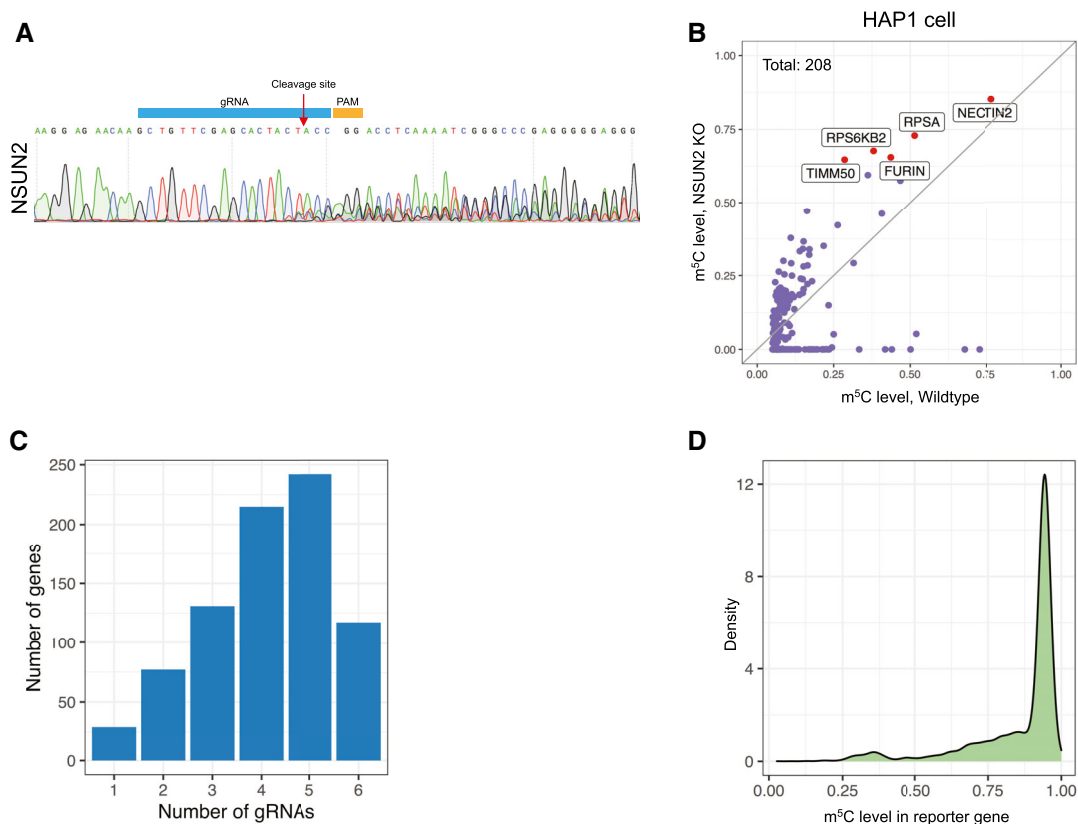


Figure EV1. NSUN2 Knockout and the effect on the reporter as well as endogenous m⁵C sites.

- A The NSUN2-KO efficiency in Cas9-expressing HAP1 cells. Seven days after viral transduction, NSUN2 was efficiently mutated.
- B Scatter plot demonstrating the effect of NSUN2 knocked out in HAP1 cells. 208 m⁵C sites were identified in wild-type HAP1 cells, 90 (43.3%) of which showed significantly reduced m⁵C level in NSUN2-KO cells. The five m⁵C sites with the highest modification rate in NSUN2-KO cells were highlighted as red dots and marked with gene names. The gray line represents the diagonal line, along which the modification rate is equal between wild-type and NSUN2 knockout cells.
- C Distribution of genes with different number of gRNAs detected.
- D Density plot of m⁵C level of the reporter site associated with individual gRNA. The Y-axis represents the density of gRNAs.

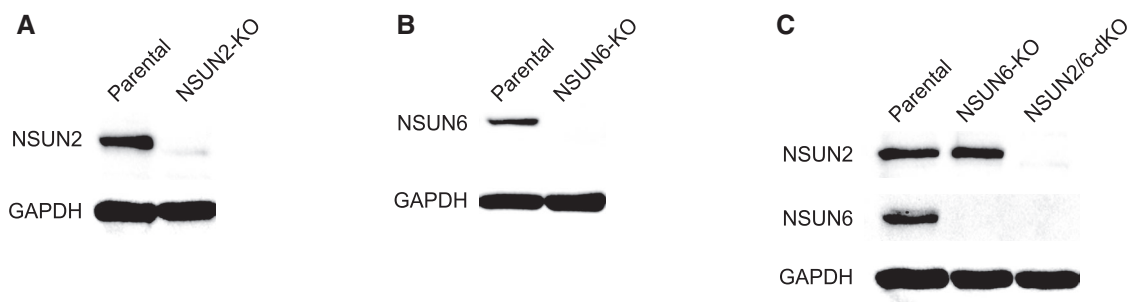


Figure EV2. KO of NSUN2 and/or NSUN6 in HAP1 cells.

- A–C Western Blot demonstrating the effect NSUN2 (A), NSUN6 (B) as well as NSUN2/6 double knockout (C). NSUN2/6 double knockout (NSUN2/6-dKO) HAP1 cells were established based on clonal NSUN6-KO cells.

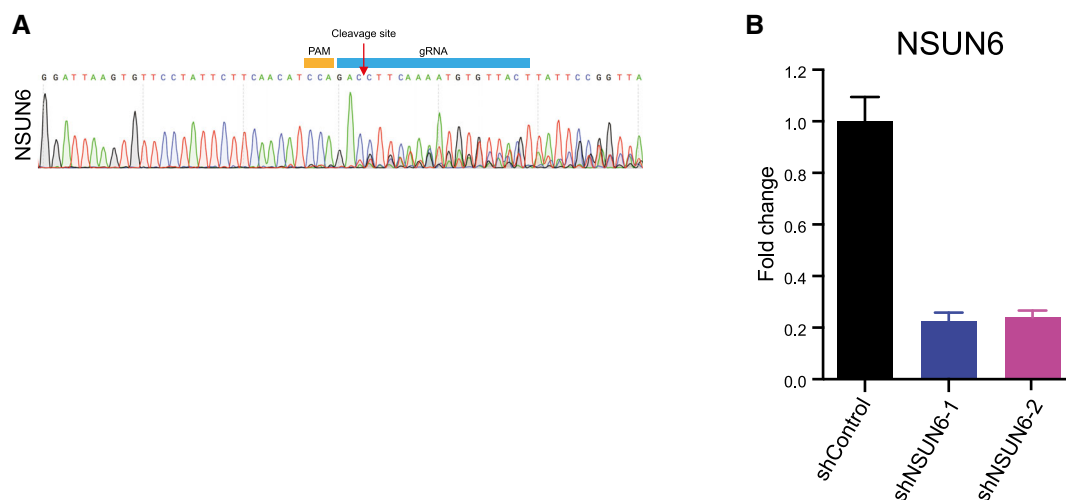


Figure EV3. NSUN6 was efficiently knocked out or knocked down.

A The KO efficiency of NSUN6 in Cas9-expressing HAP1 cells. Seven days after viral transduction, NSUN6 was efficiently mutated.
 B The knockdown efficiency of NSUN6 in HAP1 cells. Three independent biological replicates were performed. Error bars represent SD.

Figure EV4. mRNA-BisSeq profiles of the three m⁵C modification sites depend on both NSUN6 and NSUN2.

A–C IGV plots showing the m⁵C sites in ANGEL1, ZNF707, and STRN4 genes. The m⁵C sites in ANGEL1 (A) and ZNF707 (B) possessed a 3' TCCA and a 3' AGGG motif, respectively, while the m⁵C site in STRN4 was among a cluster of “pseudo” m⁵C sites (C).

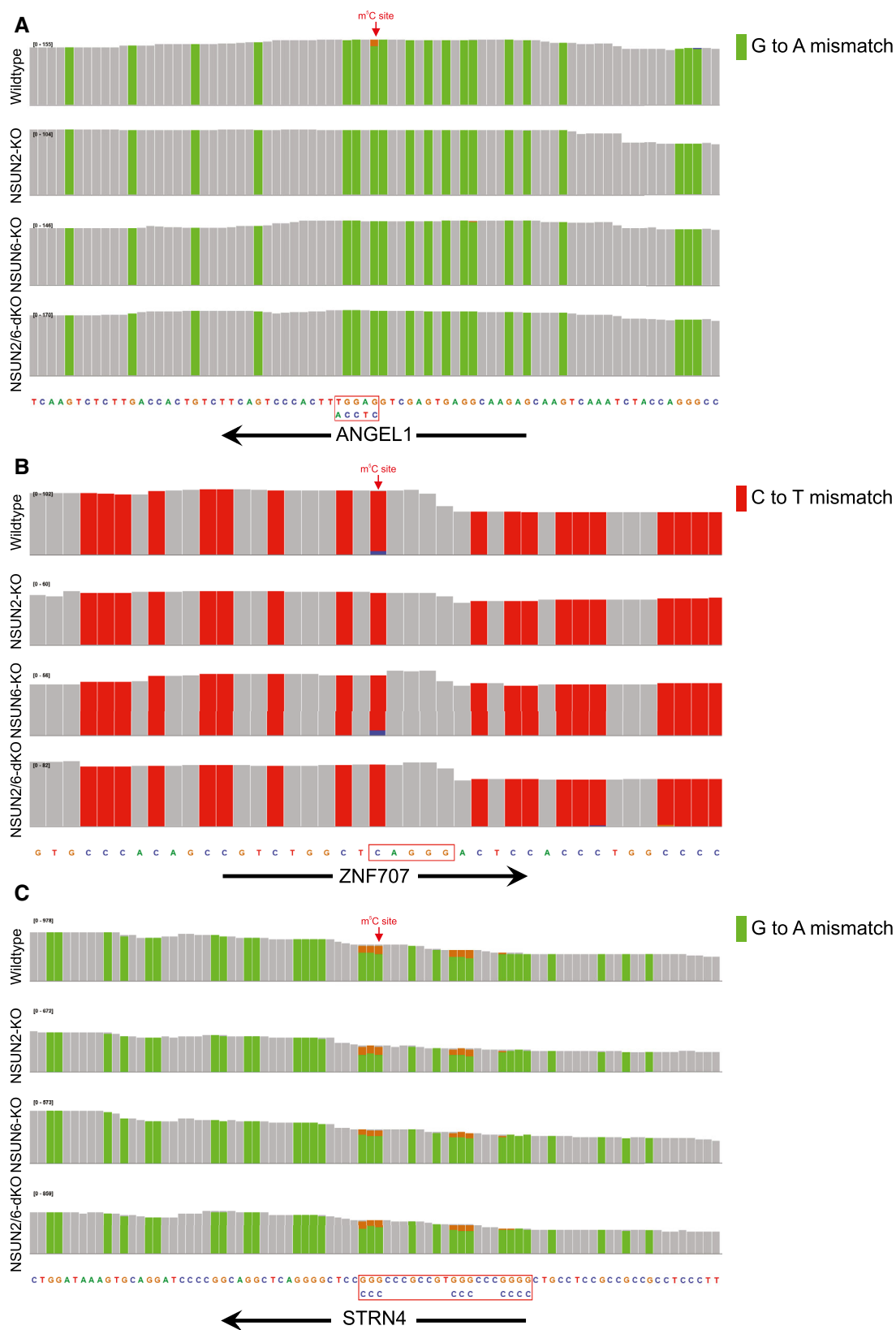


Figure EV4.

ID	position	gene	sequences	ID	position	gene	sequences
1	11@16756861@+	C11orf58		29	2@216633619@+	IGFBP2	
2	11@66687583@-	SPTBN2		30	5@180071683@-	RNF130	
3	11@767963@-	GATD1		31	5@180071687@-	RNF130	
4	11@767969@-	GATD1		32	6@113859942@+	MARCKS	
5	11@768098@-	GATD1		33	6@113859943@+	MARCKS	
6	11@768104@-	GATD1		34	6@113859945@+	MARCKS	
7	12@110281749@+	ATP2A2		35	6@149724702@-	NUP43	
8	12@110281750@+	ATP2A2		36	7@100070158@-	ZNF3	
9	12@110281752@+	ATP2A2		37	7@100070166@-	ZNF3	
10	14@74736311@+	FCF1		38	X@119538511@-	CXorf56	
11	14@74736317@+	FCF1		39	X@119538527@-	CXorf56	
12	15@65150178@-	CLPX		40	7@151080695@-	FASTK	
13	15@85234697@-	AC044860.1		41	7@151080696@-	FASTK	
14	16@1964562@-	RPS2		42	7@151080697@-	FASTK	
15	16@2972795@+	PAQR4		43	8@12755322@-	LONRF1	
16	16@50368785@-	BRD7		44	8@17156688@+	ZDHC2	
17	18@31685103@-			45	8@17156691@+	ZDHC2	
18	19@13118331@+	NACC1		46	8@17156694@+	ZDHC2	
19	19@13118340@+	NACC1		47	MT@14423@-	MT-ND6	
20	1@150308596@+	MRPS21		48	MT@1486@+	MT-RNR1	
21	1@156676704@-	NES		49	MT@1488@+	MT-RNR1	
22	1@31938230@-	PTP4A2		50	MT@8387@-		
23	20@36891800@-	SAMHD1		51	MT@8391@-		
24	20@43667277@+	MYBL2		52	MT@8392@-		
25	22@49961503@+	PIM3		53	6@42989628@+	PPP2R5D	
26	2@101253124@+	CNOT11		54	7@134293043@-	SLC35B4	
27	2@216633613@+	IGFBP2		55	5@179616893@-	HNRNPH1	
28	2@216633616@+	IGFBP2		56	5@80626854@-	DHFR	

Figure EV5. NSUN2/6-independent m⁵C sites.

The group of 56 NSUN2/6-independent sites was highly enriched for clusters of pseudo m⁵C sites: 52 sites had at least one pseudo m⁵C site in vicinity.