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Supplemental information

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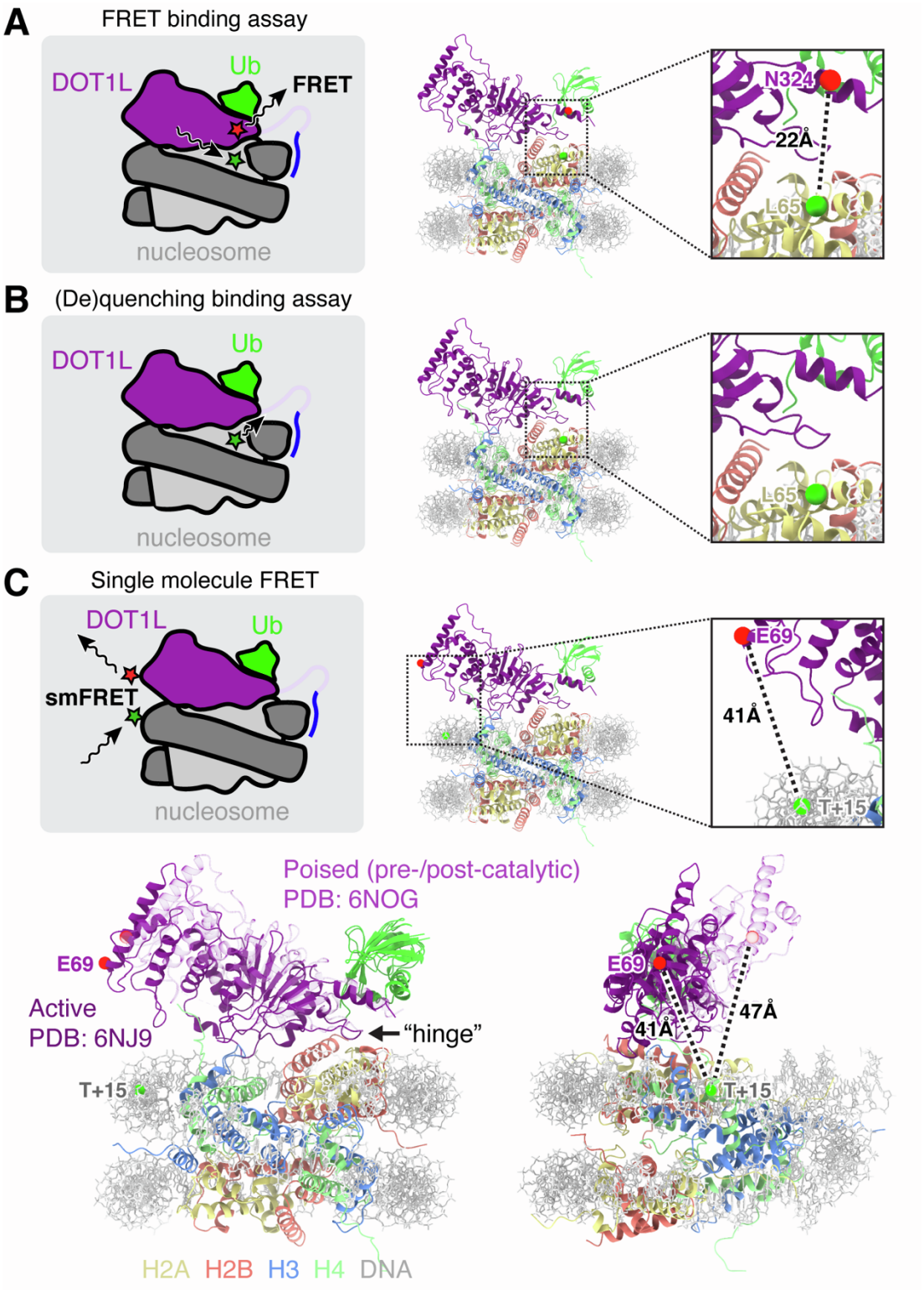

Figure S1, Related to Figures 1-3. Fluorophore labeling positions depicted on DOT1L and nucleosome. (A) Fluorophore labeling positions used in bulk FRET assay. **(B)** Fluorophore labeling positions used in (de)quenching local acidic patch binding assay. **(C)** Fluorophore labeling

positions used in single-molecule FRET experiments. Comparison of fluorophore separation distance in single-molecule FRET experiments based on “active” (PDB: 6NJ9) and “poised” (PDB: 6NOG) structures (bottom). The distances between E69 (Cα) and T+15 (N3) where the fluorophores are attached are 41 and 47 Å, respectively for the “active” and “poised” states. Interflourophore distance for all reported “poised” structures ranges from 44-47 Å.

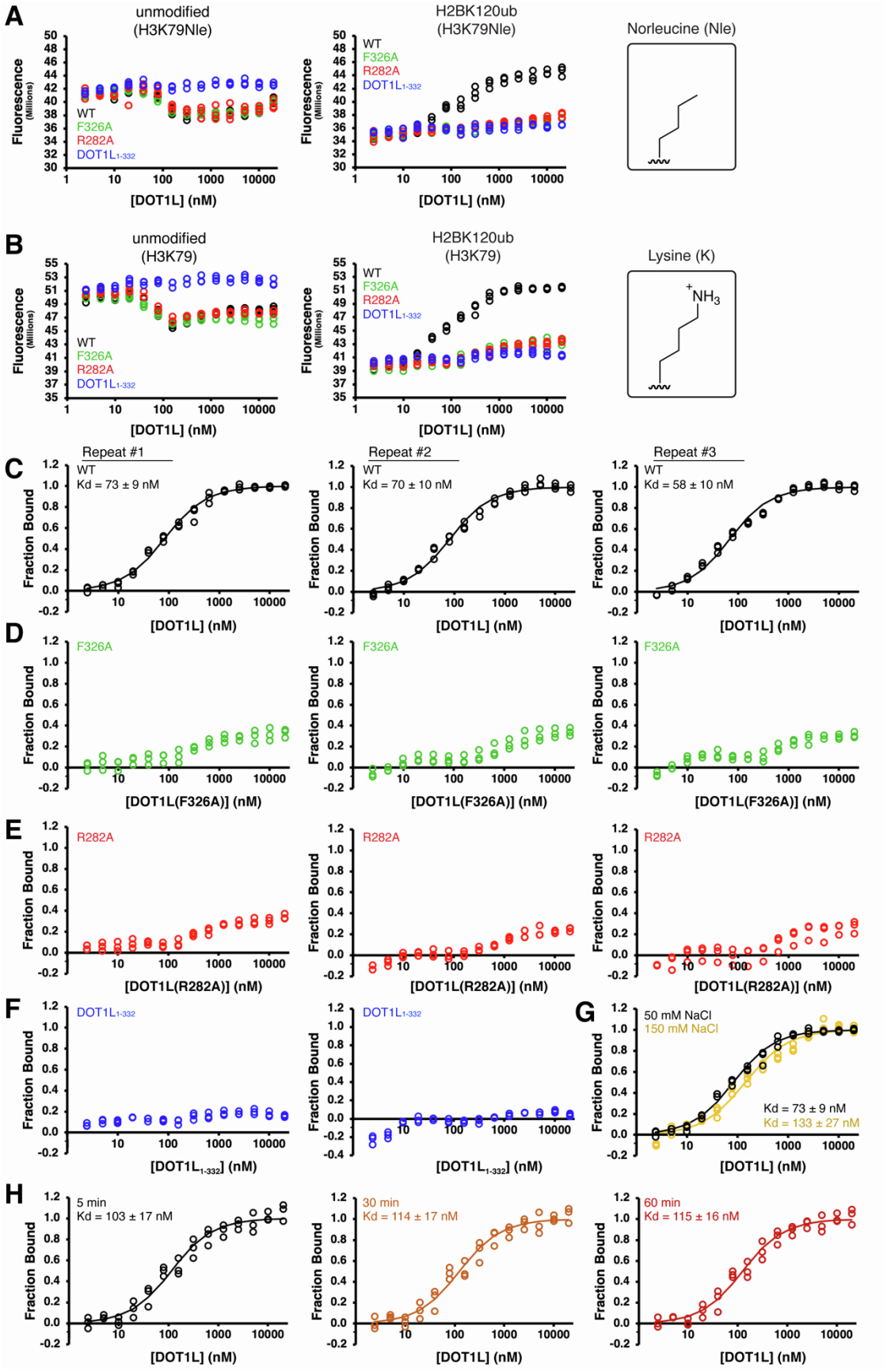
A
unmodified
(H3K79Nle)
H2BK120ub
(H3K79Nle)
Norleucine (Nle)
Fluorescence (Millions)
WT
F326A
R282A
DOT1L1-332
[DOT1L] (nM)
B
unmodified
(H3K79)
H2BK120ub
(H3K79)
Lysine (K)
NH3
C
Repeat #1
Repeat #2
Repeat #3
WT
Kd = 73 ± 9 nM
Kd = 70 ± 10 nM
Kd = 58 ± 10 nM
Fraction Bound
D
F326A
[DOT1L(F326A)] (nM)
E
R282A
[DOT1L(R282A)] (nM)
F
DOT1L1-332
[DOT1L1-332] (nM)
G
50 mM NaCl
150 mM NaCl
Kd = 73 ± 9 nM
Kd = 133 ± 27 nM
H
5 min
Kd = 103 ± 17 nM
30 min
Kd = 114 ± 17 nM
60 min
Kd = 115 ± 16 nM

Figure S2, Related to Figure 2 and STAR Methods. (De)quenching binding assay quantification and replicability. Raw fluorescence data for DOT1L mutants binding to unmodified (left) and H2BK120ub nucleosomes (right) containing **(A)** H3K79Nle substitution or **(B)** or wild type H3K79. **(C-F)** Comparison of independently run replicate experiments for binding to H2BK120ub wild type nucleosomes using **(C)** wild type DOT1L, **(D)** DOT1L(F326A), **(E)** DOT1L(R282A), or **(F)** $DOT1L_{1-332}$. **(G)** Comparison of wild type DOT1L binding to H2BK120ub H3K79 nucleosomes at 50 mM or 150 mM NaCl shows salt dependence of binding. **(H)** (De)quenching binding assay timecourse demonstrates that equilibrium is reached within 30 min of reagent combination. Individual data points are shown for three independent titrations.

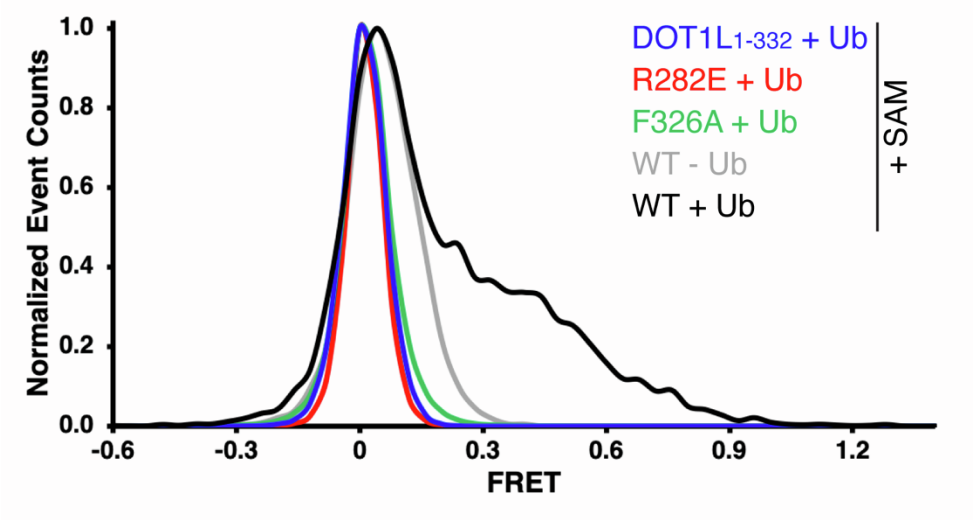

Figure S3, Related to Figure 3. H2BK120ub not required for low-FRET state. Overlays of normalized single-molecule FRET histograms for DOT1L binding to unmodified or H2BK120ub nucleosomes, all in the presence of SAM.

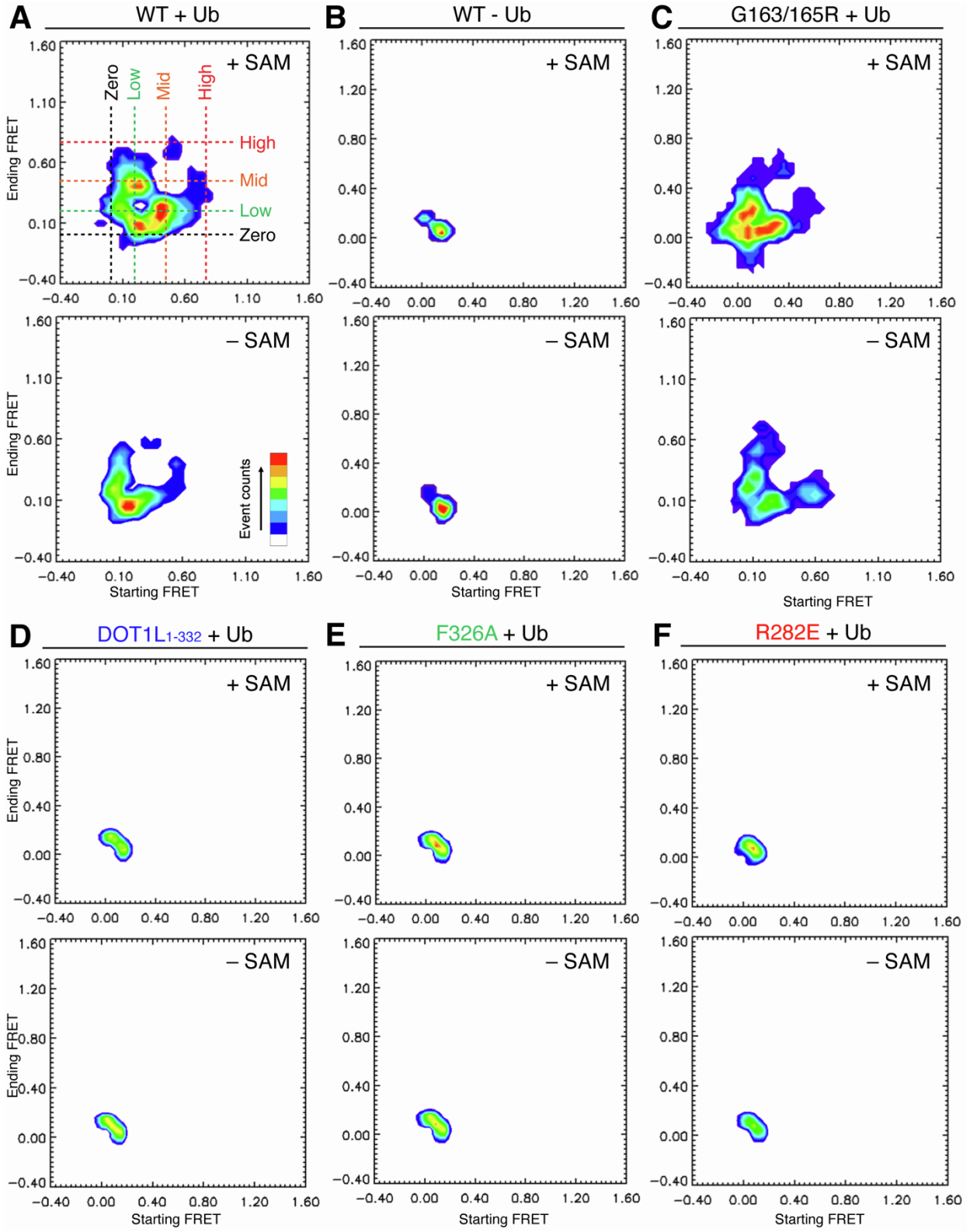

Figure S4, Related to Figure 4. Heat maps of single-molecule FRET transitions. Single-molecule FRET heat maps displaying starting versus ending FRET of individual events for wild type DOT1L$_{cat}$ (WT) binding to **(A)** H2BK120ub (n = 161 and 141 smFRET time trajectories,

respectively, for with and without SAM) or **(B)** unmodified nucleosomes (n = 1251 and 1047, respectively, for with and without SAM). Single-molecule FRET heat maps displaying starting versus ending FRET of individual events for H2BK120ub nucleosomes and **(C)** DOT1L G163/165R (n = 123 and 88, respectively, for with and without SAM), **(D)** $DOT1L_{1-332}$ (n = 868 and 2178, respectively, for with and without SAM), **(E)** DOT1L F326A (n = 2882 and 2885, respectively, for with and without SAM), or **(F)** DOT1L R282E (n = 3348 and 4768, respectively, for with and without SAM). Results are shown for experiments performed in the presence (top) or absence of SAM (bottom).

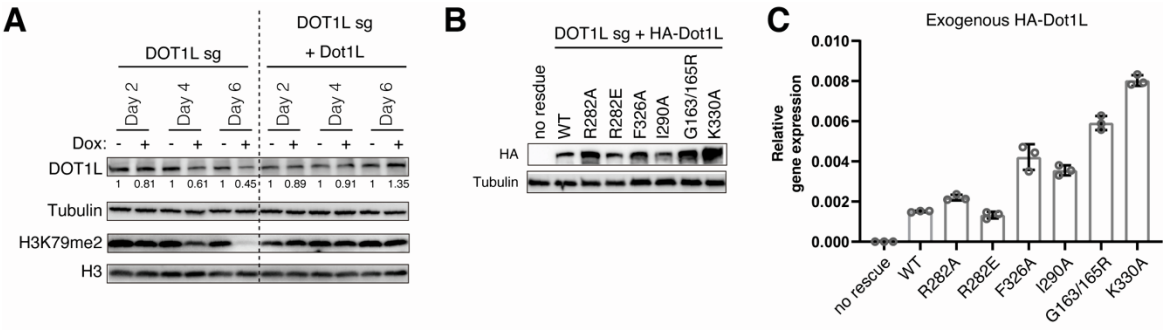

Figure S5, Related to Figure 5. Exogenous Dot1L expression in MV4;11 cells. (A) Quantitation of DOT1L levels in western blots from Fig. 5 relative to Dox – samples on same day using Image J. DOT1L antibody detects both endogenous DOT1L and exogenous murine Dot1L. **(B)** Western blot and **(C)** RT-qPCR for exogenous HA-tagged murine Dot1L (+ HA-Dot1L), either wild type (WT) or the indicated mutant, in MV4;11 cells with endogenous DOT1L depleted by CRISPR/Cas9 (DOT1L sg) (n = 3 biological replicates; presented as mean ± SD).

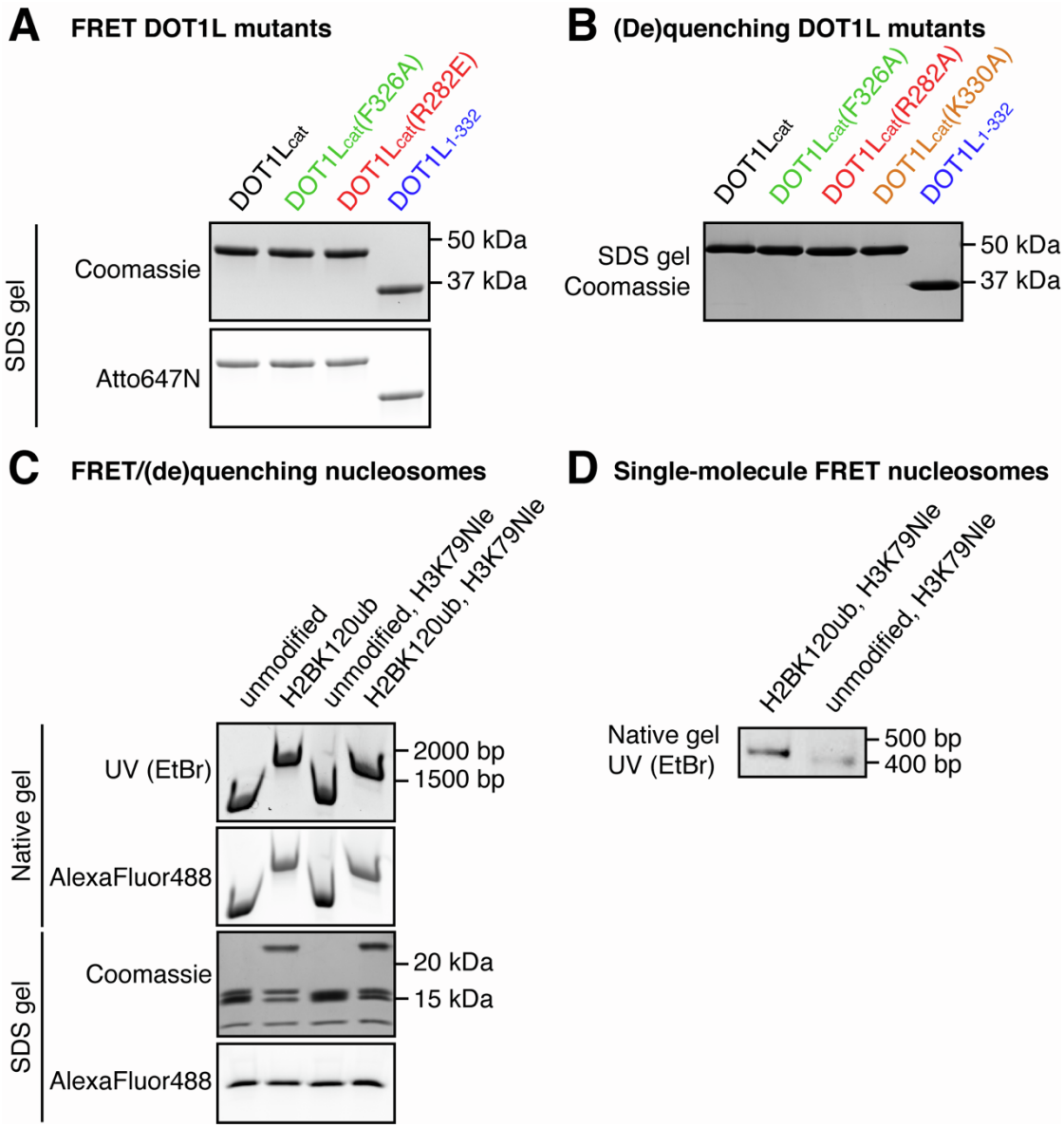

Figure S6, Related to STAR Methods. DOT1L and nucleosome reagents. DOT1L proteins used in **(A)** bulk FRET binding assays and **(B)** (de)quenching local binding assays run on SDS denaturing gels. **(C)** Fluorescently labelled nucleosomes used in FRET and (de)quenching assays run on 10% native acrylamide and SDS denaturing gels. **(D)** Nucleosomes used in single-molecule FRET experiments run on 5% native acrylamide gel. Atto647N and AlexaFluor488 fluorescent scans were recorded using a GE Typhoon FLA 9000 Gel Imaging Scanner. EtBr = ethidium bromide.

Name	*Base Sequence*	
F1	/5Biosg/ GCATGTAAGT GCATGTAAGT ATCGAGAATC CCGGTGCCGA GGCCGCTCAA TTGG	54
F2	/5Phos/ TCGTAGACAG CTCTAGCACC GCTTAAACGC ACGTACGCGC TGTCCCCCGC GTTT /iAmMC6T/AACCG CCAAGGGGAT TAC	73
F3	/5Phos/ TCCCTAGTCT CCAGGCACGT GTCAGATATA TACATCCGAT	40
R1	ATCGGATGTA TATATCTGAC ACGTGCCTGG AGACTAGGGA GTAATCCCCT TGGCGGTTAA AACGCGGGGG	70
R2	/5Phos/ACAGCGCGTA CGTGCGTTTA AGCGGTGCTA GAGCTGTCTA CGACCAATTG AGCGGCCTCG GCACCGGGAT TCTCGAT	77

Table S1, Related to STAR Methods. DNA fragments used to form Widom 601 nucleosome positioning sequence for smFRET measurments.

Nucleosome	*DOT1L*	K_D	*Hill Coefficient*
unmodified	WT	32 ± 1 nM	2.4 ± 0.2
unmodified	F326A	38 ± 1 nM	2.6 ± 0.1
unmodified	R282E	57 ± 3 nM	2.4 ± 0.2
H2BK120ub	WT	36 ± 2 nM	2.6 ± 0.2
H2BK120ub	F326A	36 ± 5 nM	1.7 ± 0.1
H2BK120ub	R282E	53 ± 3 nM	2.4 ± 0.2

Table S2, Related STAR Methods. Bulk FRET binding assay fit values.

Gene	*Forward primer*	*Reverse primer*
mDot1L	CAGAGGATGACCTGTTTGTCG	CATCCACTTCCTGAACTCTCG
hPBX3	GCCTTGGAGGAAATTCACTG	AGATGGAGTTGTTGCGTCCT
hMEIS1	GGGCATGGATGGAGTAGGC	GGGTACTGATGCGAGTGCAG
hHOXA10	AGGTGGACGCTGCGGCTAATCTCTA	GCCCCTTCCGAGAGCAGCAAAG
hGAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

Table S3, Related to STAR Methods. RT-qPCR primers.