

Supplementary Material

Vitamin D3 ameliorates R-loop-induced replication stress and chromosomal instability in MED12-mutant uterine fibroids

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Figure S1

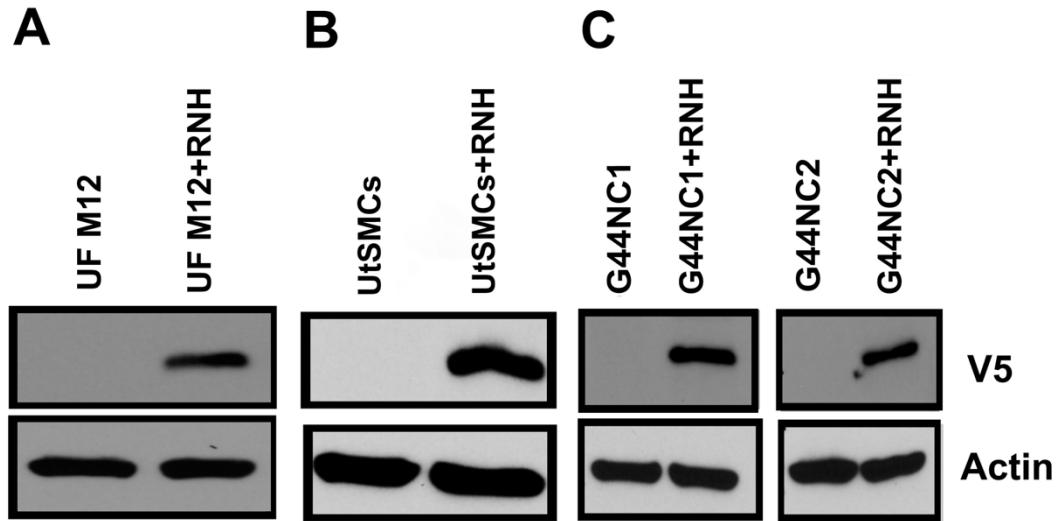


Figure S1. Validation of ectopic RNaseH1 expression in cell culture models. (A) Primary MED12-mutant (M12) UFs were transduced with V5-tagged RNase H1 (RNH)-expressing lentivirus in the presence 2 μ g/mL of polybrene. 48h post-transduction, cells were processed for immunoblot analysis using antibodies specific for the V5 epitope and β -actin, the latter of which served as an internal loading control. (B) Parental and V5-tagged WT RNase H1 (RNH)-expressing immortalized uterine smooth muscle cell (UtSMC) lines were processed by immunoblot analysis using antibodies specific for the V5 epitope and β -actin. (C) Two different CRISPR-engineered MED12-mutant G44N clones (C1 and C2) were transduced with V5-tagged RNase H1 (RNH)-expressing lentivirus in the presence 2 μ g/mL of polybrene. 48h post-transduction, cells were processed for immunoblot analysis using antibodies specific for the V5 epitope and β -actin.

Figure S2

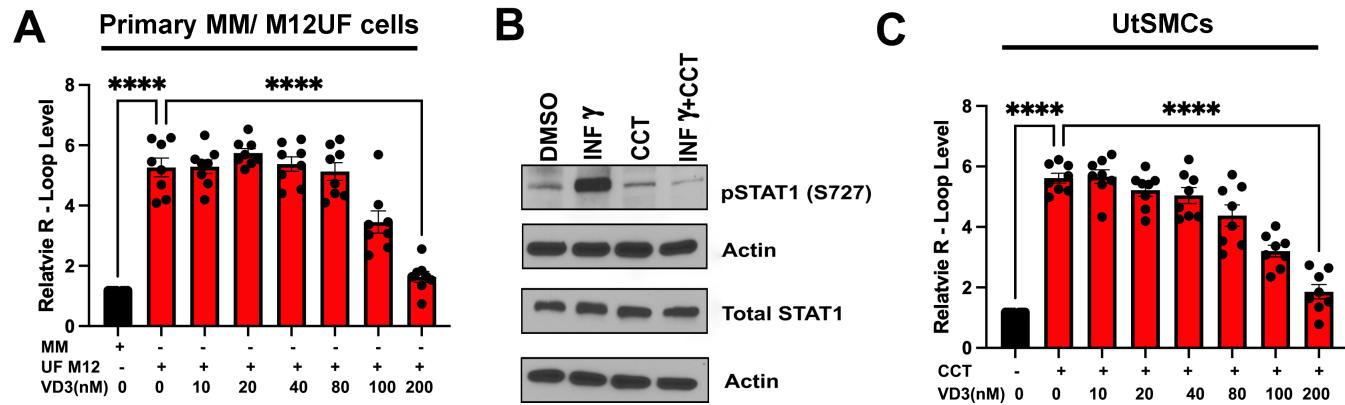


Figure S2. VD3 dose-dependently suppresses R-loop accrual in primary MED12-mutant UFs and Mediator kinase inhibited UtSMCs. (A) Quantification of R-loop levels in primary MM and MED12-mutant (M12) UFs based on immunocytochemical analysis using the RNA-DNA hybrid-specific antibody S9.6. Where indicated, cells were treated without (-) or with (+) VD3 (0-200nm). Data are plotted as fold-change in S9.6 antibody signal intensity relative to untreated MM cells. Quantification was determined from a minimum of n=50 cells in each of 4 technical replicates from 2 independent experiments (n \geq 200 total cells) per treatment condition. **(B)** Validation of Mediator kinase (CDK8/19) inhibition by CCT251545 in UtSMCs. As indicated, UtSMCs were pre-treated for 24h with DMSO or CCT251545 (CCT: 100nM) prior to 45min treatment without or with interferon gamma (INF γ ; 10ng/ml) to stimulate Mediator kinase activity. Thereafter, treated cells were harvested and processed for immunoblot analysis using antibodies specific for phosphorylated STAT1^{S727} (pSTAT1), bulk STAT1, and β -ACTIN, the latter of which served as an internal loading control. As shown by us and others, STAT1^{S727} is a validated biomarker of Mediator kinase activity (1-3). **(C)** Quantification of R-loop levels in UtSMCs based on S9.6-based immunocytochemical analysis. Where indicated, UtSMCs were treated without (DMSO) or with the Mediator kinase (CDK8/19) inhibitor (CCT; 100nM) and Vit D3 (VD3; 0-200nM). Data are plotted as fold-change in S9.6 antibody signal intensity relative to untreated UtSMCs. Quantification was determined from a minimum of n=50 cells in each of 4 technical replicates from 2 independent experiments (n \geq 200 total cells) per treatment condition. Significance calculated using One-Way ANOVA followed Tukey's Post hoc test, ****p< 0.0001, *p<0.05.

Figure S3

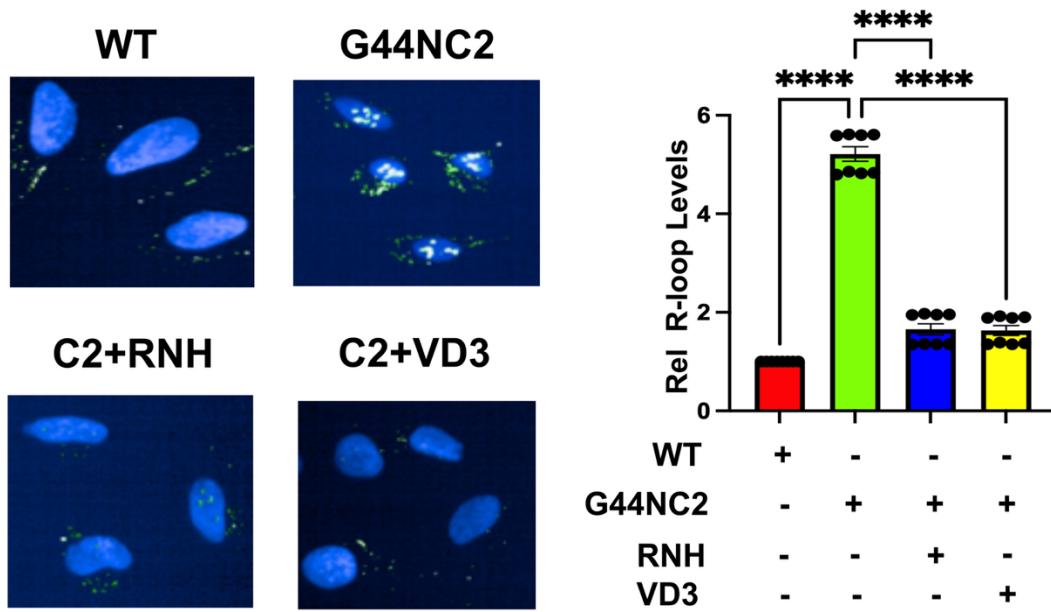


Figure S3. VD3 suppresses R-loop accrual in MED12-mutant (G44N) hTERT cells.
Representative images (*left*) and quantification (*right*) of R-loop levels in parental MED12 WT hTERT-immortalized uterine smooth muscle (WT) cells and their derivative CRISPR-engineered MED12-mutant G44N hTERT (G44N) cells. R-loop levels were determined by immunocytochemical analysis using the RNA-DNA hybrid-specific antibody S9.6. Two different CRISPR-engineered G44N clones (C1 and C2) were analyzed. Clone 1 is shown in Figure 3. Clone 2 is shown here. Where indicated, G44NC2 cells were treated with VD3 (100nM) or transduced with RNase H1 (RNH)-expressing lentivirus. Data are plotted as fold-change in S9.6 antibody signal intensity relative to parental MED12 WT cells. Quantification was determined from a minimum of n=50 cells from each of 4 technical replicates from 2 independent experiments (n \geq 400 total cells) per treatment condition. Significance calculated using One-Way ANOVA followed Tukey's Post hoc test, ****p < 0.0001, ***p < 0.001.

Figure S4

A

	Control n = 10	VD3 0.1 µg/kg/day n = 3	VD3 0.5 µg/kg/day n = 3	DCL 0.3 µg/kg/day n = 4	p value
AST (U/L)					
Mean ± SD	222.4 ± 139.7	165.7 ± 170.0	236.3 ± 208.2	349.5 ± 215.1	0.4846
ALT (U/L)					
Mean ± SD	30.91 ± 10.02	32.33 ± 13.65	34.67 ± 11.59	30.75 ± 5.9	0.9384
Total Bilirubin (mg/dL)					
Mean ± SD	0.20 ± 0.04	0.23 ± 0.05	0.16 ± 0.05	0.22 ± 0.09	0.4188
Calcium (mg/dL)					
Mean ± SD	9.91 ± 0.57	9.53 ± 1.29	10.17 ± 2.17	11.93 ± 1.65	0.1865

B

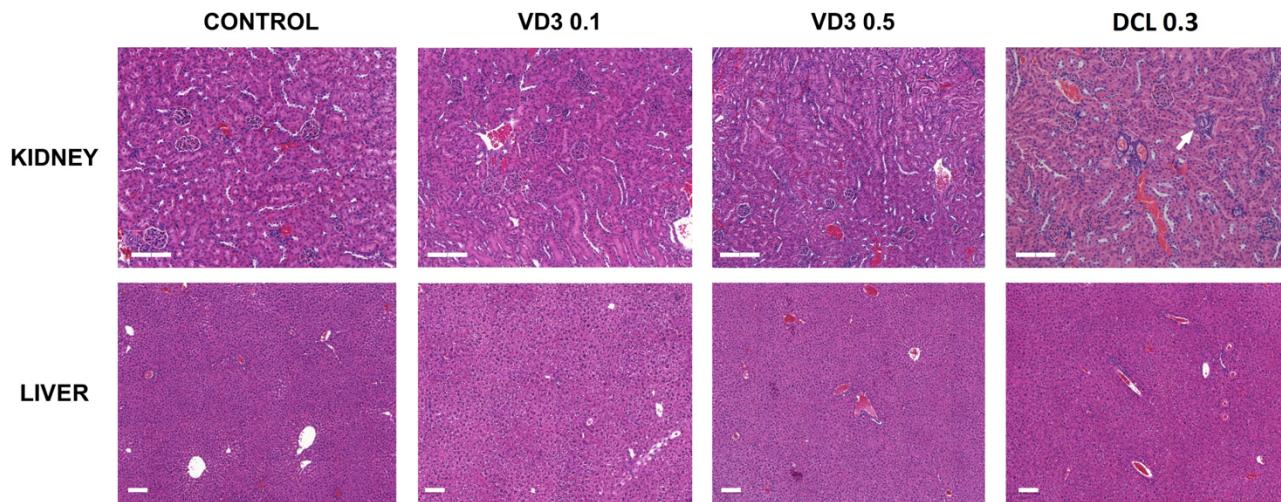


Figure S4. Safety profiles of mice treated with Vitamin D (VD3) and Doxercalciferol (DCL). **(A)** Serum levels of liver function markers Aspartate Aminotransferase (AST), Alanine Transaminase (ALT) total Bilirubin, and calcium in mice treated with vehicle (CONTROL), VD3 0.1 µg/kg/day (VD3 0.1), VD3 0.5 µg/kg/day (VD3 0.5) and DCL0.3 µg/kg/day (DCL 0.3). Values represented as mean ± standard deviation (SD). **(B)** Representative images of hematoxylin and eosin staining for histological evaluation of liver and kidney of mice from CONTROL, VD3 0.1, VD3 0.5 and DCL 0.3 groups. The white arrow points to the presence of calcifications. Scale bars: 200 µm.

Figure S5

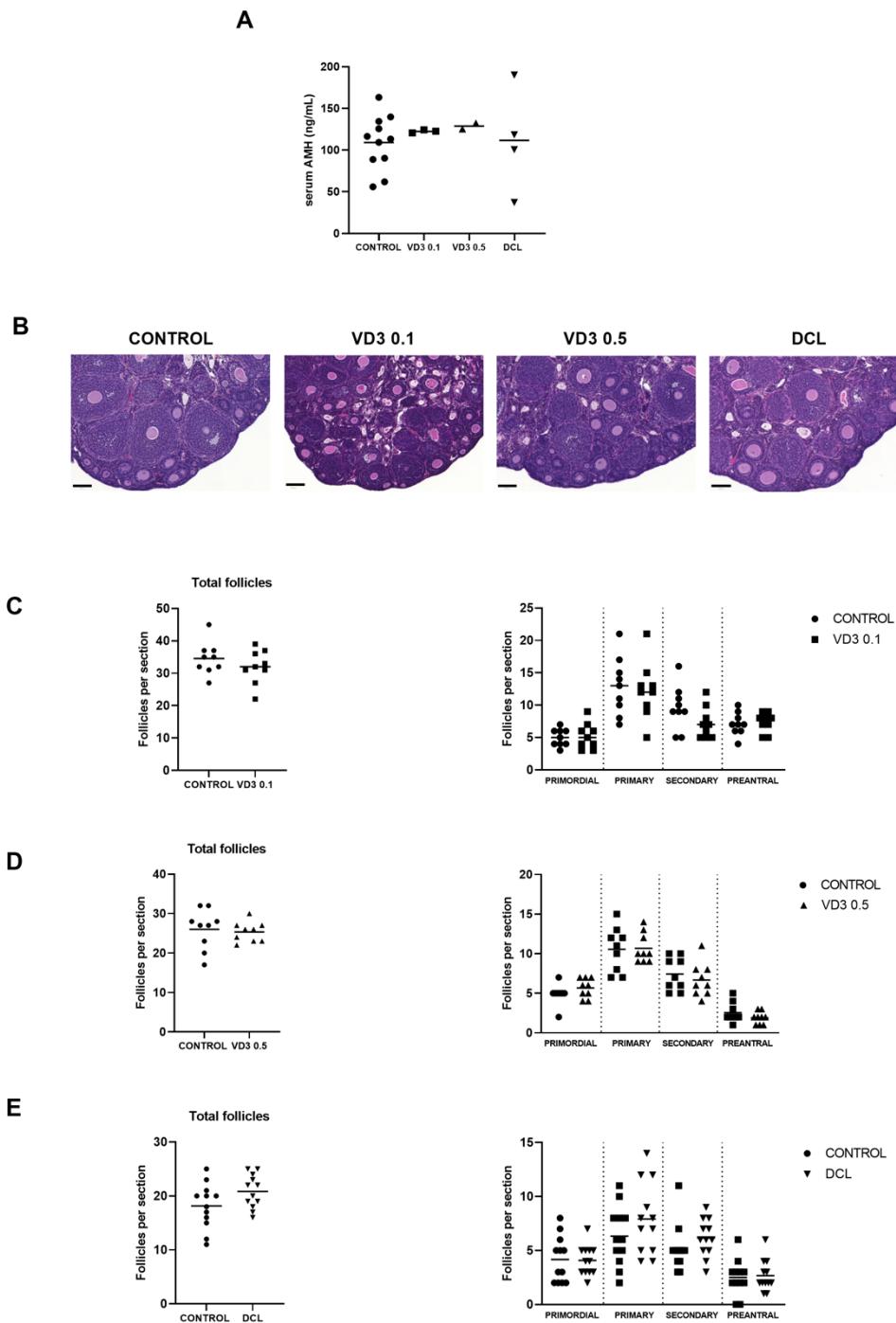


Figure S5. Evaluation of fertility potential in mice treated with Vitamin D (VD3) and Doxercalciferol (DCL). (A) Serum levels of Anti-Mullerian Hormone (AMH) in mice treated with vehicle (CONTROL), VD3 0.1 μ g/kg/day (VD3 0.1), VD3 0.5 μ g/kg/day (VD3 0.5) and DCL 0.3 μ g/kg/day (DCL 0.3). Values represented as mean \pm standard deviation (SD) (B) Representative images of hematoxylin and eosin staining for histological evaluation of mouse ovaries from CONTROL, VD3 0.1, VD3 0.5 and DCL 0.3 groups. (C-E) Number of total follicles and primordial, primary, secondary and preantral follicles in mice treated with VD3 0.1 (C), VD3 0.5 (D) and DCL (E) compared to control. Scale bars: 100 μ m.

Figure S6.

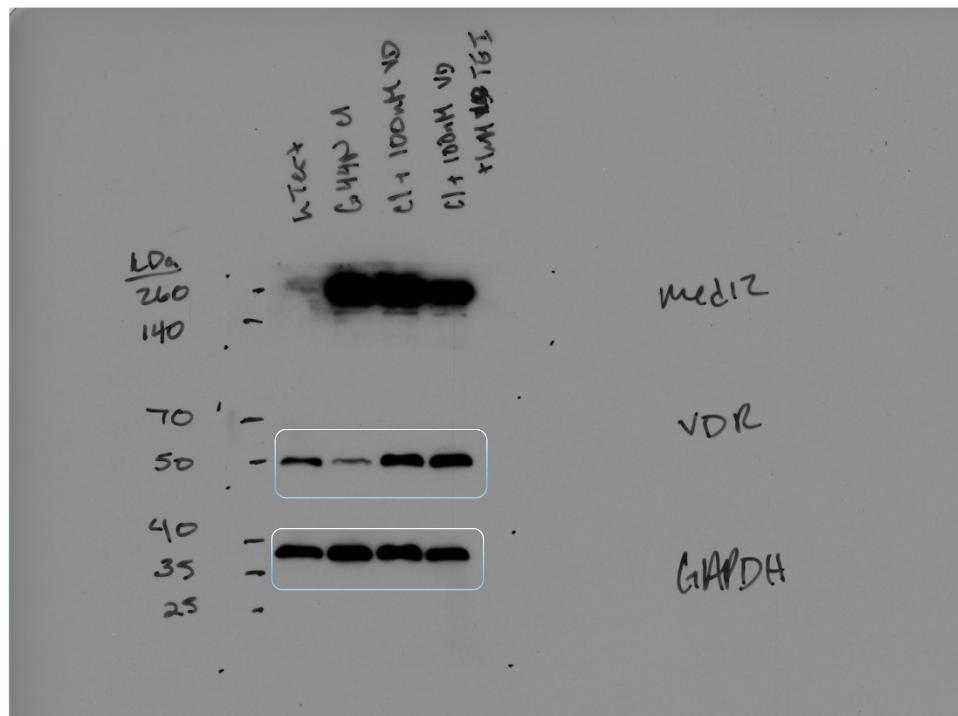


Figure S6. Related to Figure 3C. Full immunoblot showing VDR expression in WT (hTERT) and MED12 mutant (G44N) clone 1 (C1) cells.

Figure S7

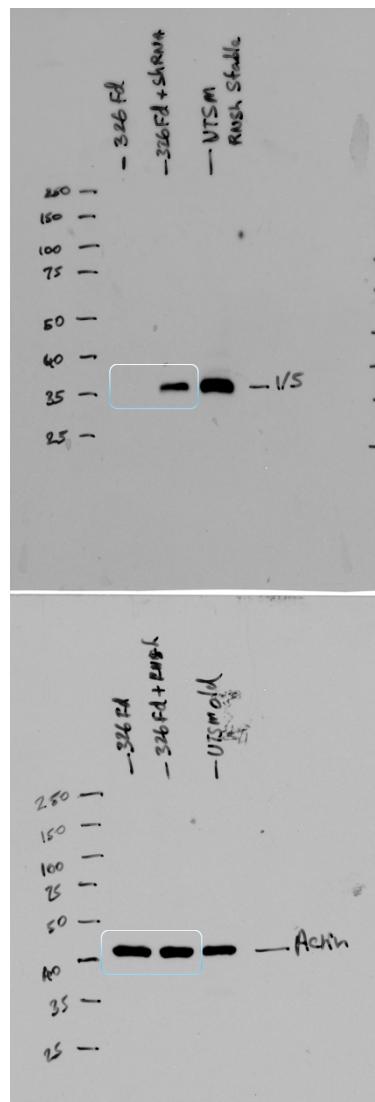


Figure S7. Related to Figure S1A. Full blot showing validation of ectopic V5-tagged RNaseH1 expression in primary MED12-mutant (M12) UFs.

Figure S8.

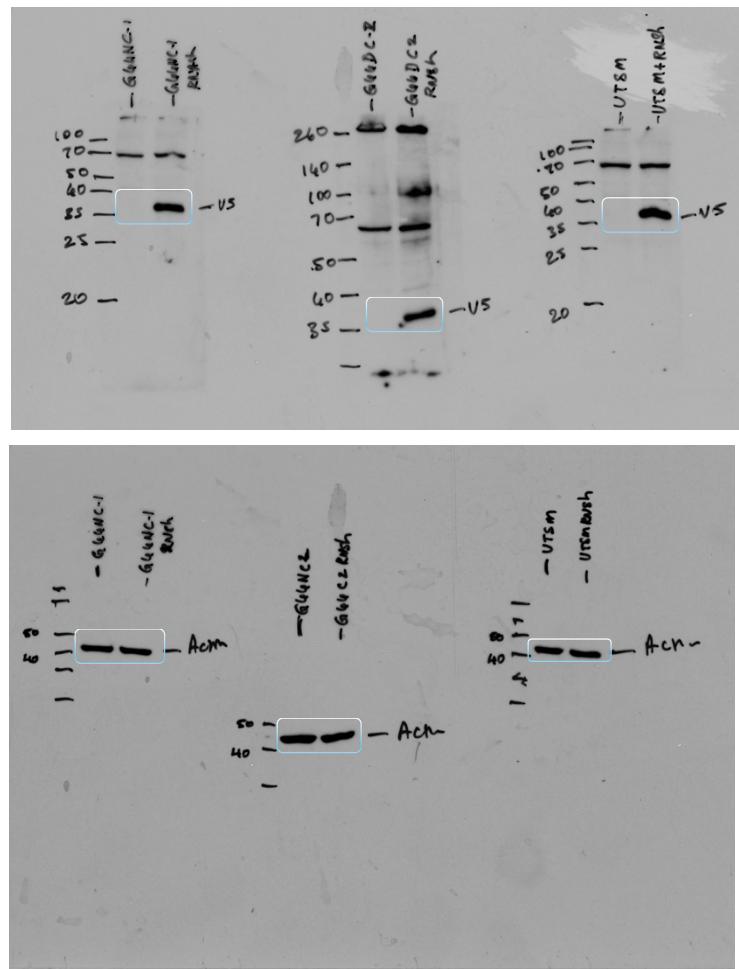


Figure S8. Related to Figure S1B and C. Full blot showing validation of ectopic V5-tagged RNaseH1 expression in UtSMCs and MED12-mutant G44N clone 1 (C1) and clone 2 (C2).

Figure S9.

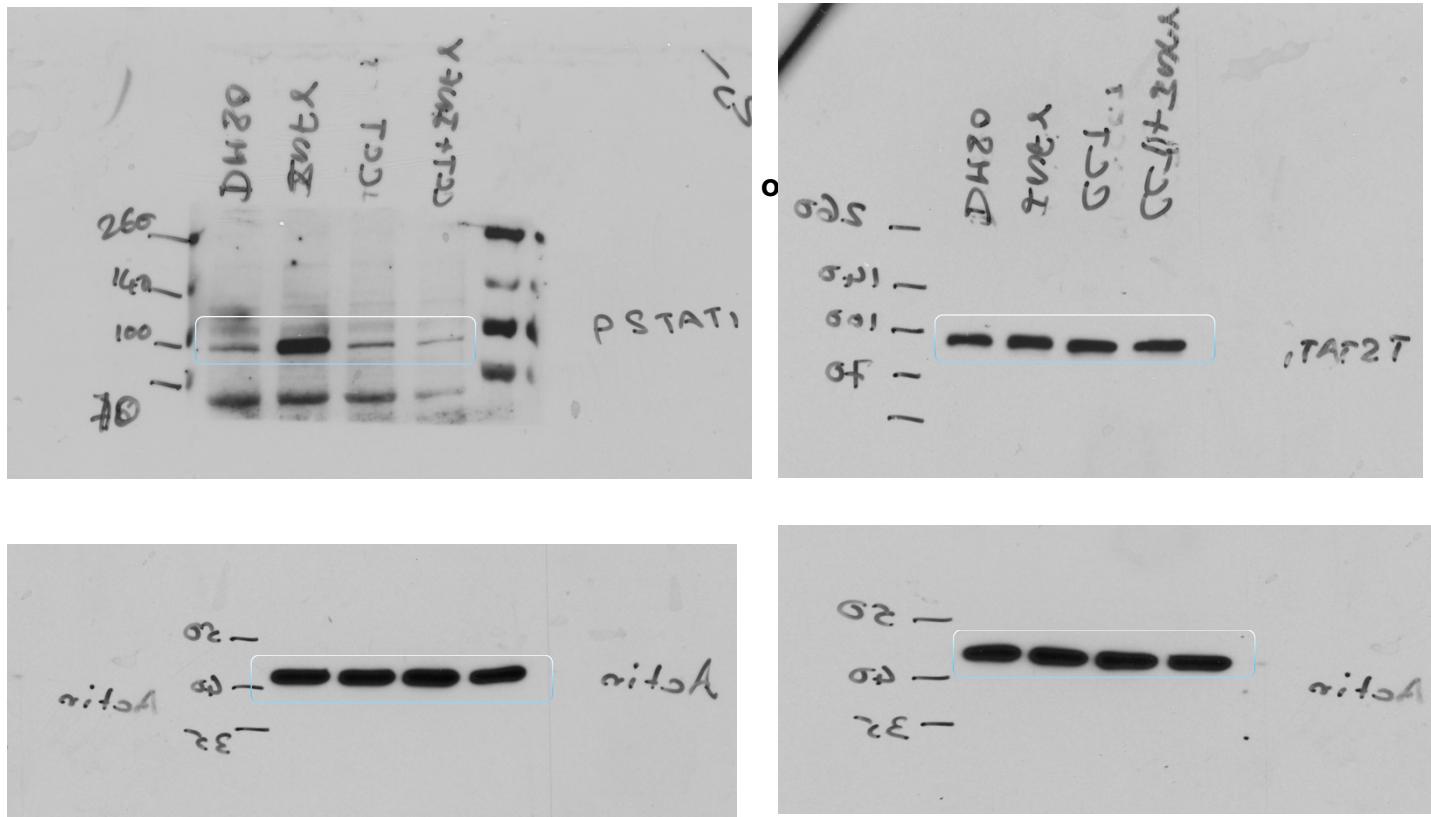


Table S1: Information relevant to patient tissue samples used for in vitro studies

Sample Number	Ethnicity	Age	Solitary/Multiple fibroids	Sample ID	MM	UF M12 status
1	Non-Hispanic or Non-Latino	29	Multiple	251	Med12 WT	L36R
2	Non-Hispanic or Non-Latino	52	Multiple	255	Med12 WT	G44D
3	Non-Hispanic or Non-Latino	49	Multiple	262	Med12 WT	G44D
4	Non-Hispanic or Non-Latino	71	Multiple	264	Med12 WT	G44D
5	Unknown	37	Multiple	266	Med12 WT	G44S
6	Hispanic or Latino	69	Multiple	296	Med12 WT	G44S
7	Hispanic or Latino	43	Multiple	299	Med12 WT	G44D
8	Non-Hispanic or Non-Latino	39	Multiple	314	Med12 WT	G44V
9	Hispanic or Latino	49	Multiple	338	Med12 WT	G44S
10	Non-Hispanic or Non-Latino	46	Multiple	347	Med12 WT	G44V

Table S2: Information relevant to patient tissue samples used for mouse PDX studies

Sample Number	Ethnicity	Age	Sample ID	UF M12 status
1	African American	45	UCTB44	G44R
2	African American	43	UCTB 52	G44D
3	African American	43	UCTB 56	G44D
4	African American	38	UCTB 88	G44D
5	African American	36	UCTB 90	G44D
6	African American	51	UCTB 91	c.100_120del21
7	African American	36	UCTB 97	G44S
8	African American	48	UCTB 110	G44D
9	African American	35	UCTB 111	G44D
10	African American	48	UCTB 113	G44D

Supplementary References

1. Barron L, Khadka S, Schenken R, He L, Blenis J, Blagg J, et al. Identification and characterization of the Mediator kinase-dependent myometrial stem cell phosphoproteome. *F&S Science*. 2021;2(4):282-295.
2. Dale T, Clarke PA, Esdar C, Waalboer D, Adeniji-Popoola O, Ortiz-Ruiz MJ, et al. A selective chemical probe for exploring the role of CDK8 and CDK19 in human disease. *Nature chemical biology*. 2015;11(12):973-80.
3. Poss ZC, Ebmeier CC, Odell AT, Tangpeerachaikul A, Lee T, Pelish HE, et al. Identification of Mediator Kinase Substrates in Human Cells using Cortistatin A and Quantitative Phosphoproteomics. *Cell Rep*. 2016;15(2):436-50.