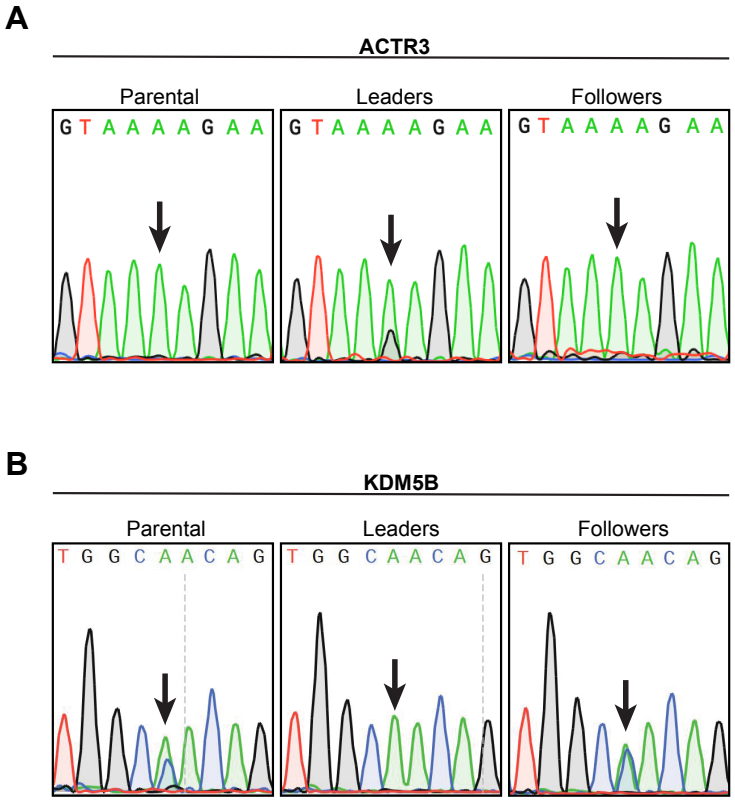

Fig. S1. Confirmation of leader- and follower-enriched mutations. Sanger sequencing confirming leader-enriched ACTR3 mutation (A) and follower-enriched KDM5B mutation (B) in cDNA (shown) and genomic DNA isolated from H1299 parental, leader and follower populations. Black arrows indicate the bases of interest. (A) Only the wild-type A peak is seen in the parental and follower populations, while the leader population contains both A and G peaks. (B) Only the wild-type A peak is seen in the leader population, while the parental and follower populations contain both A and C peaks.

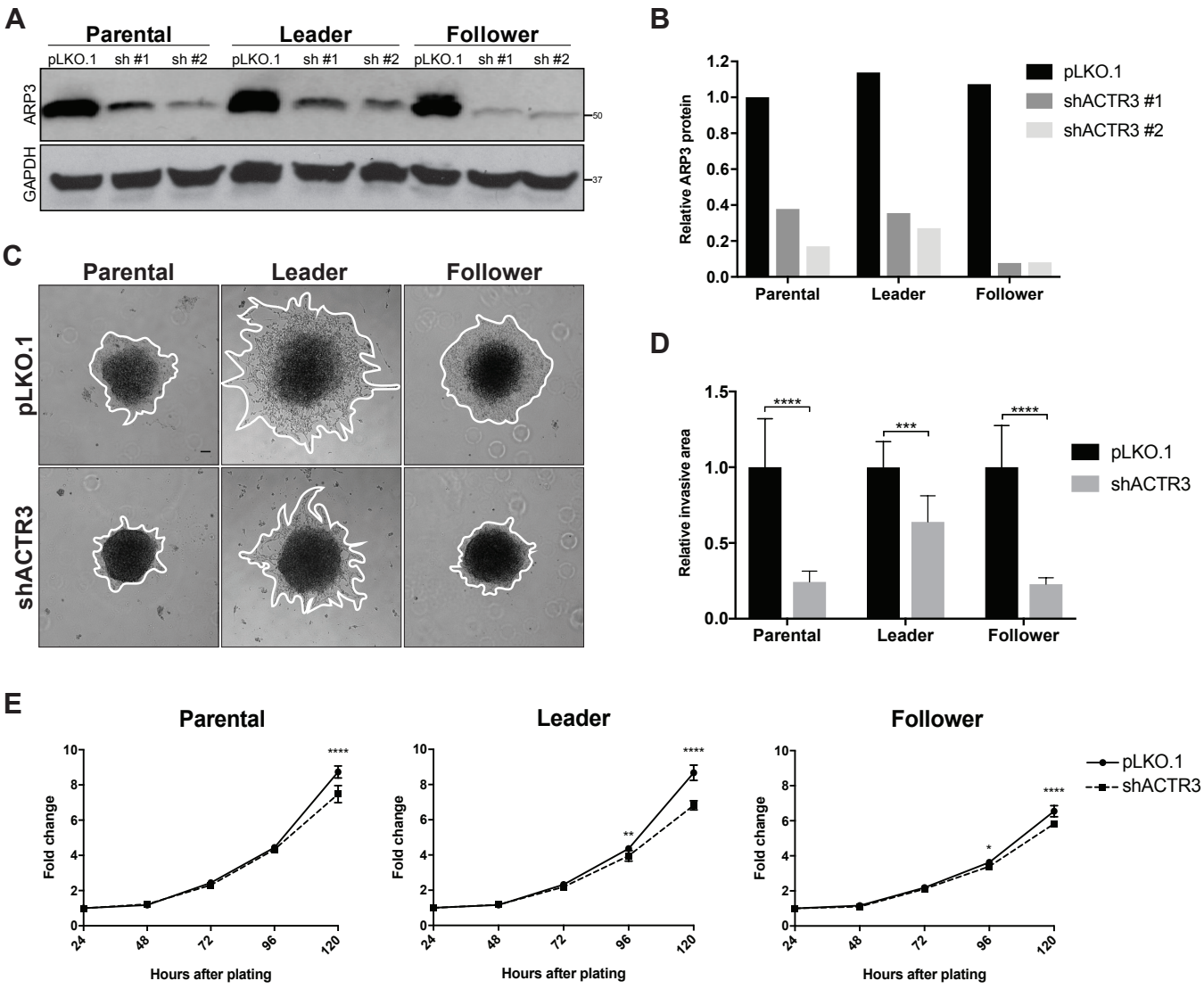

Fig. S2. ARP3 knockdown inhibits 3-D invasion. (A) Western blot showing ARP3 protein levels in H1299 parental, leader and follower cells upon expression of empty pLKO.1 vector, ARP3 shRNA #1 (Millipore Sigma TRCN0000029383), or ARP3 shRNA #2 (Millipore Sigma TRCN0000380403). (B) Western blot densitometry quantification, indicating 70-90% knockdown of ARP3 protein using either shRNA #1 or shRNA #2. (C) Representative images of 24-hour invasion of H1299 parental, leader, and follower spheroids expressing either empty pLKO.1 or shACTR3 #2. Scale bar = 100μm. (D) Quantification of relative 24-hour invasive area, normalized to pLKO.1 control for each group. (mean±s.d., n=5, 11, and 5 spheroids for parental, leader and follower lines, respectively. ***p<0.001, ****p<0.0001 by two-way ANOVA with Sidak correction). (E) Growth rate of parental, leader, and follower lines expressing either empty pLKO.1 or shACTR3 #2. (mean+s.d., n=5 replicates per time point. *p<0.05, ***p<0.001, ****p<0.0001 by two-way ANOVA with Šidák correction).

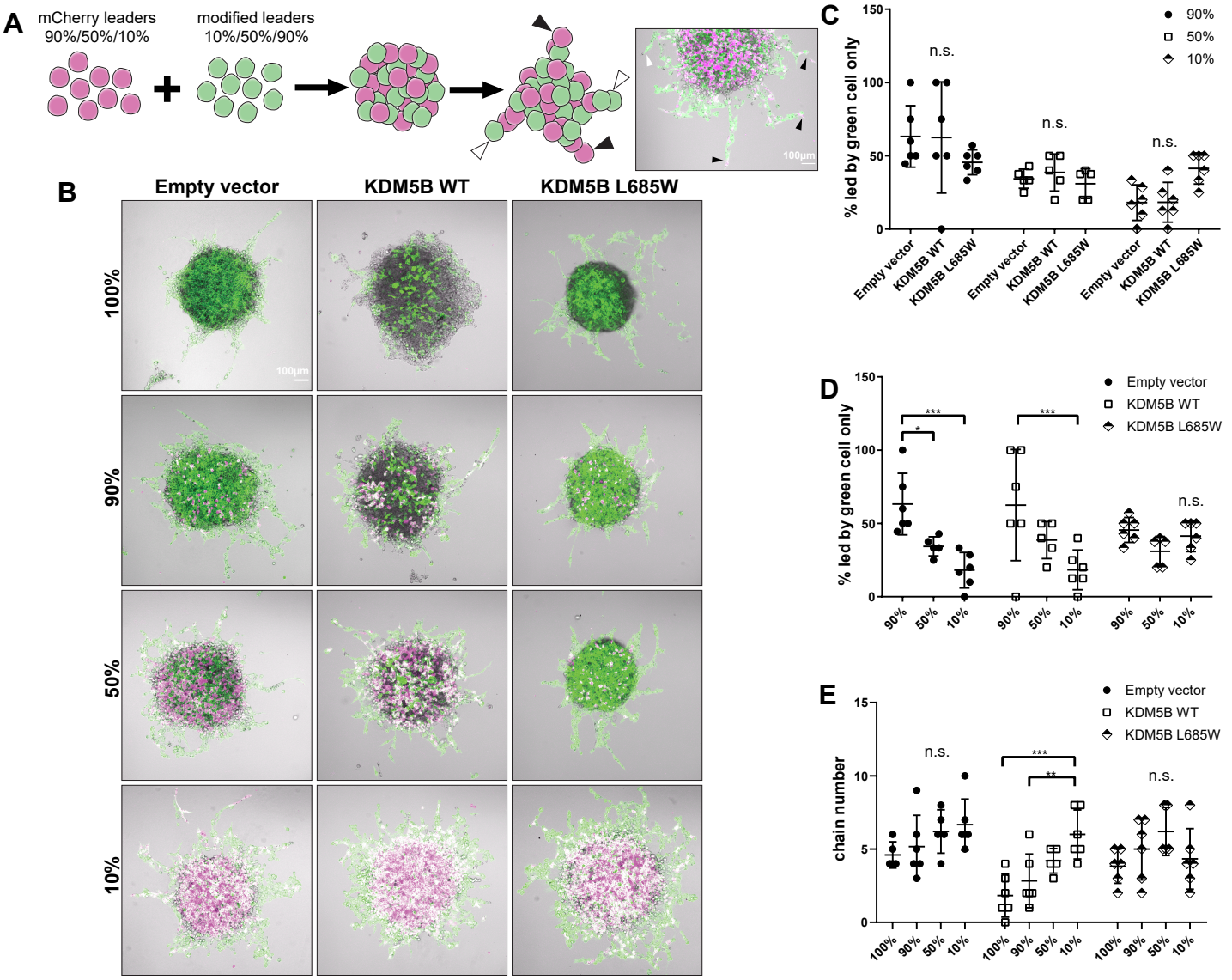

Figure S3. Leader cells overexpressing wild-type KDM5B but not KDM5B L685W exhibit diminished chain formation but retain leader activity in leader-only spheroids. (A) Schematic of spheroid mixing experiment in which unmodifed mCherry leaders were mixed with empty vector, KDM5B wild-type, or KDM5B L685W leaders and representative image of a spheroid containing 50% empty vector expressing and 50% unmodified leaders. Black arrows indicate magenta cell (unmodified leader) led chains. White arrows indicate green-only led chains. Both populations express Dendra2 (green) whereas the unmodified leaders also express mCherry (magenta). (B) Representative confocal fluorescence imaging of spheroids in which, 10%, 50%, or 90% leaders stably expressing empty vector, wild-type HA-KDM5B, or HA-KDM5B L685W were mixed with unmodified leaders 24 hours after embedding in Matrigel. (C) Percent invasive chains led by green-only (vector/KDM5B/KDM5B L685W) transduced cells, grouped by KDM5B overexpression cell line (mean±s.d., n=6 spheroids for 90% and 10% mixes or n=5 spheroids for 50% mixes across N=1 biological replicate, n.s. not significant, p>0.05, by two-way ANOVA with Tukey's post-test). (D) Same data as in panel B except grouped by the fraction of green-only (vector/KDM5B/KDM5B L685W) transduced cells in the spheroid (mean±s.d., n=6 spheroids for 90% and 10% mixes or n=5 spheroids for 50% mixes across N=1 biological replicate, n.s. not significant, *p<0.05, ***p<0.001 by two-way ANOVA with Tukey's post-test). (E) Average number of chains per spheroid grouped by percentage of the indicated trans-duced leader cell line (mean±s.d., n=6 spheroids for 90% and 10% mixes or n=5 spheroids for 50% mixes across N=1 biological replicate, n.s. not significant (p>0.05), **p<0.01, ***p<0.001 by two-way ANOVA with Tukey's post-test).

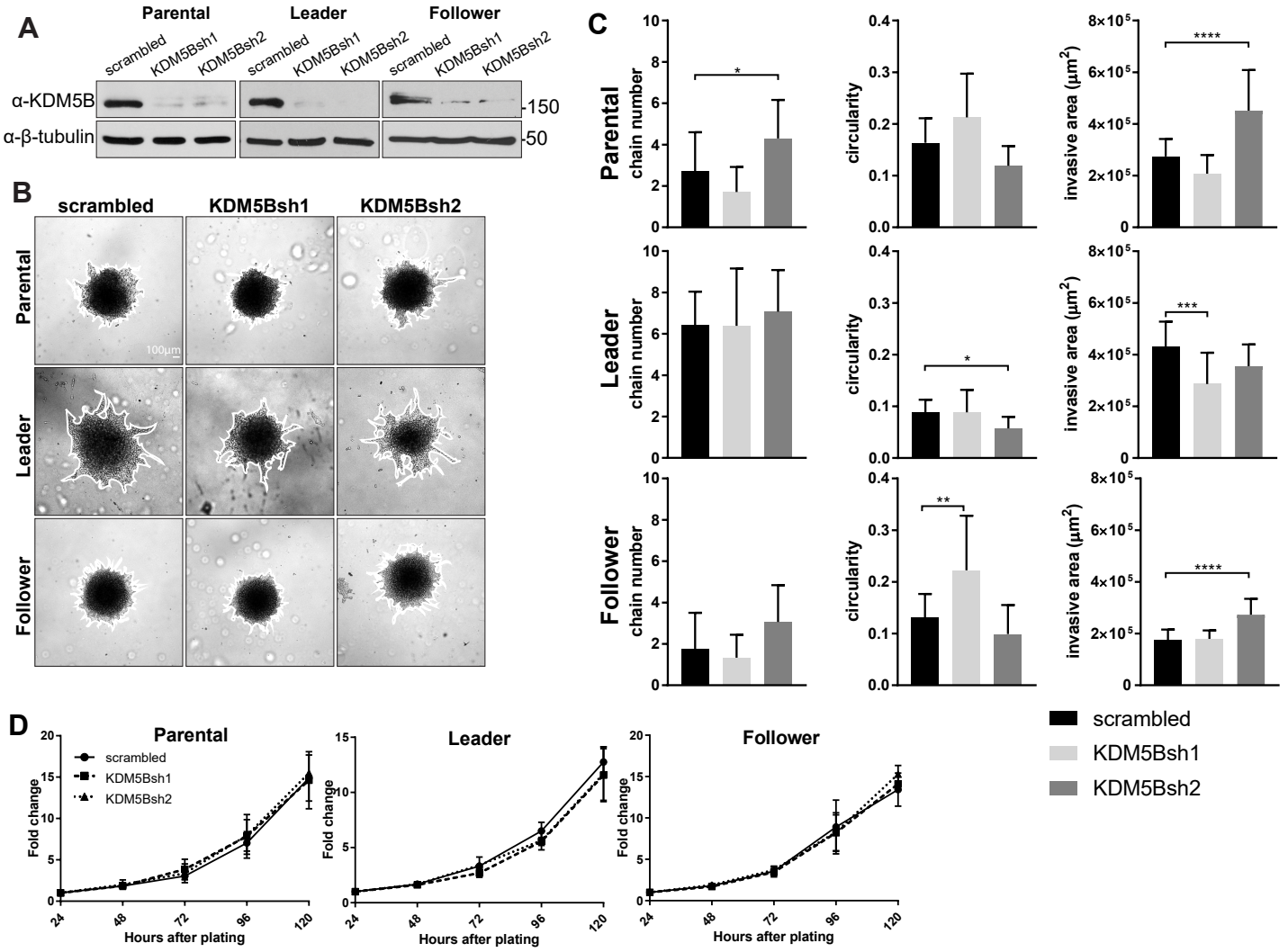

Figure S4. Impact of KDM5B knockdown on growth and chain-like invasion. (A) Western blot of KDM5B in parental, leader, and follower cell populations with two different shRNAs, shRNA1, shRNA2 against KDM5B or a scrambled control. (B) Parental, leader, and follower cells expressing scrambled shRNA, KDM5Bsh1, or KDM5Bsh2 were grown as 3-D spheroids in Matrigel at 24 hours. (C) Quantification of invasive area, circularity, and chain number from spheroids depicted in (B) (mean±s.d., *n*=13 spheroids (follower scrambled), *n*=14 spheroids (leader KDM5Bsh2), *n*=15 spheroids (leader scrambled and follower KDM5Bsh1), *n*=17 spheriods (parental scrambled, KDM5Bsh1, and KDM5Bsh2 and follower KDM5Bsh2), or *n*=18 spheroids (leader KDM5Bsh2) across *N*=3 separate experiments, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 by one-way ANOVA with Tukey's post-test). (D) Growth of parental, leader, and follower H1299 lines stably expressing scrambled shRNA, KDM5Bsh1, or KDM5Bsh2 (mean±s.d of triplicate determinations from *N*=3 independent experiments).

Table S1: PCR primers for *ACTR3* and *KDM5B*

		Primer sequence (5'-3')	
		ACTR3	KDM5B
gDNA	Forward	GTTACTTTTGTTTCTTTGTTTTTCAG	ATGTTTGTCTTGGGCTGGTG
	Reverse	TTCATATTTGCTGCTGAATACTTTT	TCAGCCCTAGAACTGCGGTA
cDNA	Forward	TCCCTCCAGAACAATCCTTG	GTCCGTAAATTGGGAGTGATTG
	Reverse	GGTTGTGTAAAGTCTGGATTAGCA	CTTTGCCTCCAAAGCTTCATTC