ALKBH4-dependent Demethylation of the Novel Actin K84me1 Modification Regulates Actomyosin Dynamics

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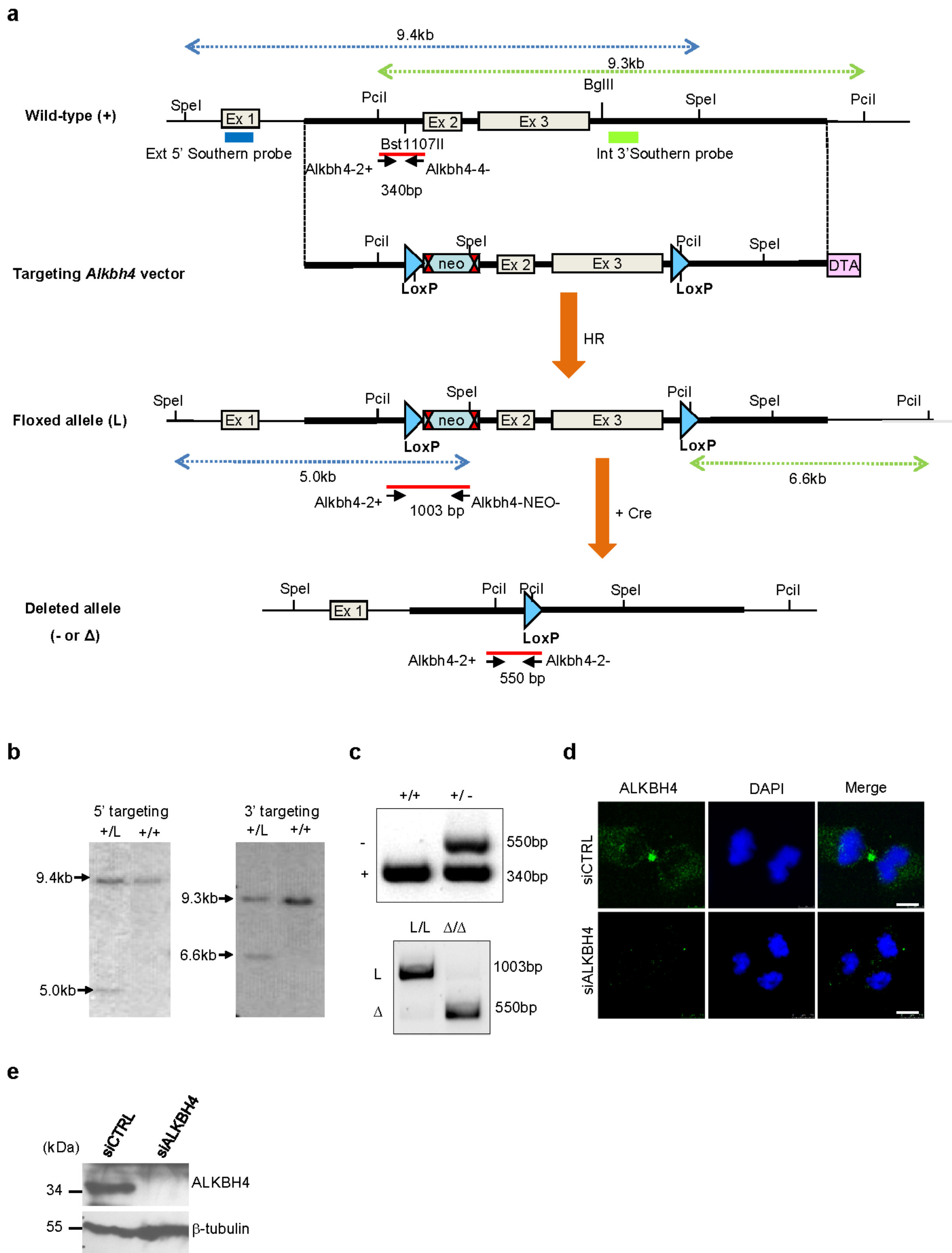
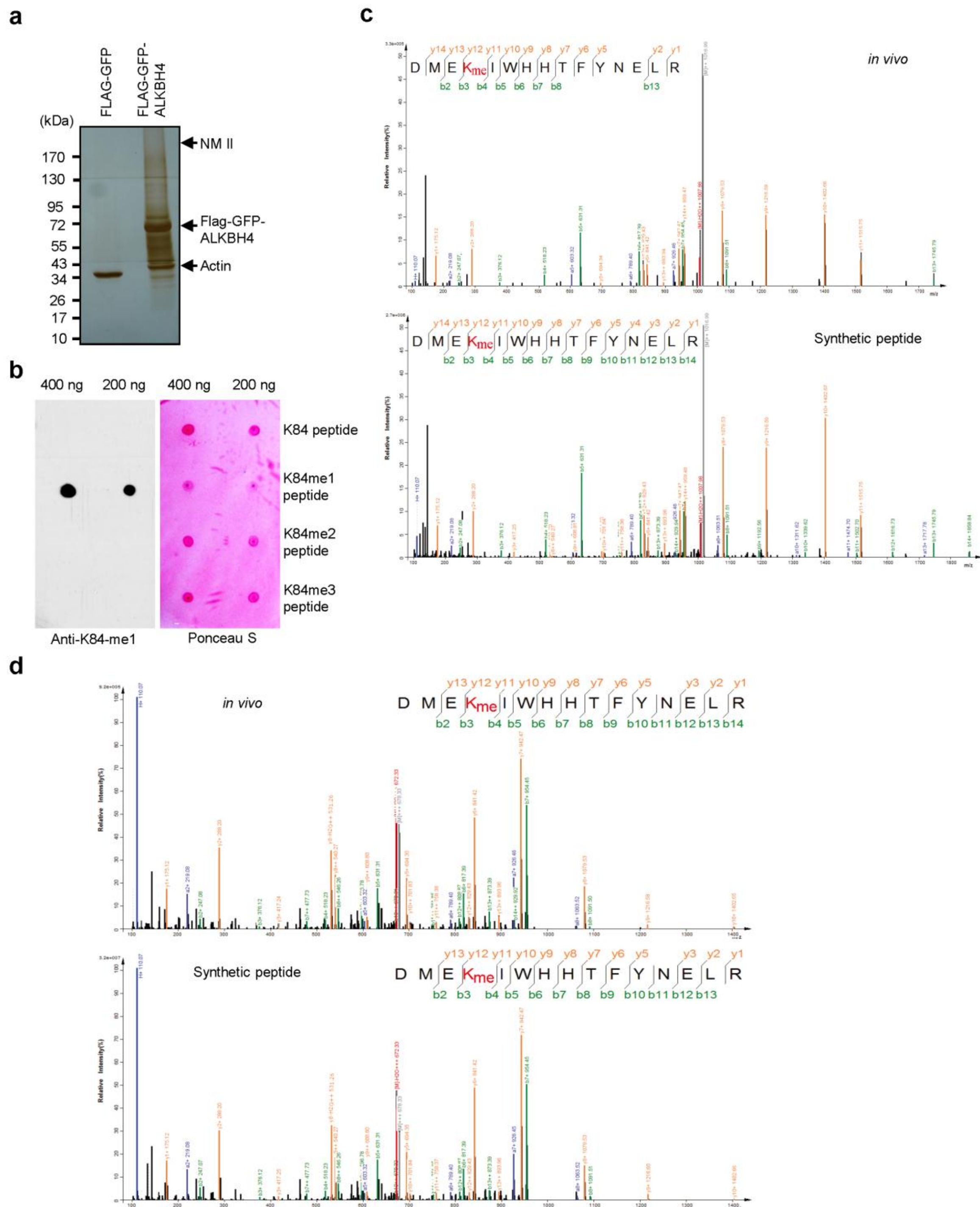


Supplementary Figure S1. Strategies for *Alkbh4* knock-out and genotyping and ALKBH4 antibody testing. a, Schematic presentation of *Alkbh4* gene-targeting and genotyping strategy, showing wild-type allele (WT); gene-targeting vector; floxed allele (L); and the Cre mediated

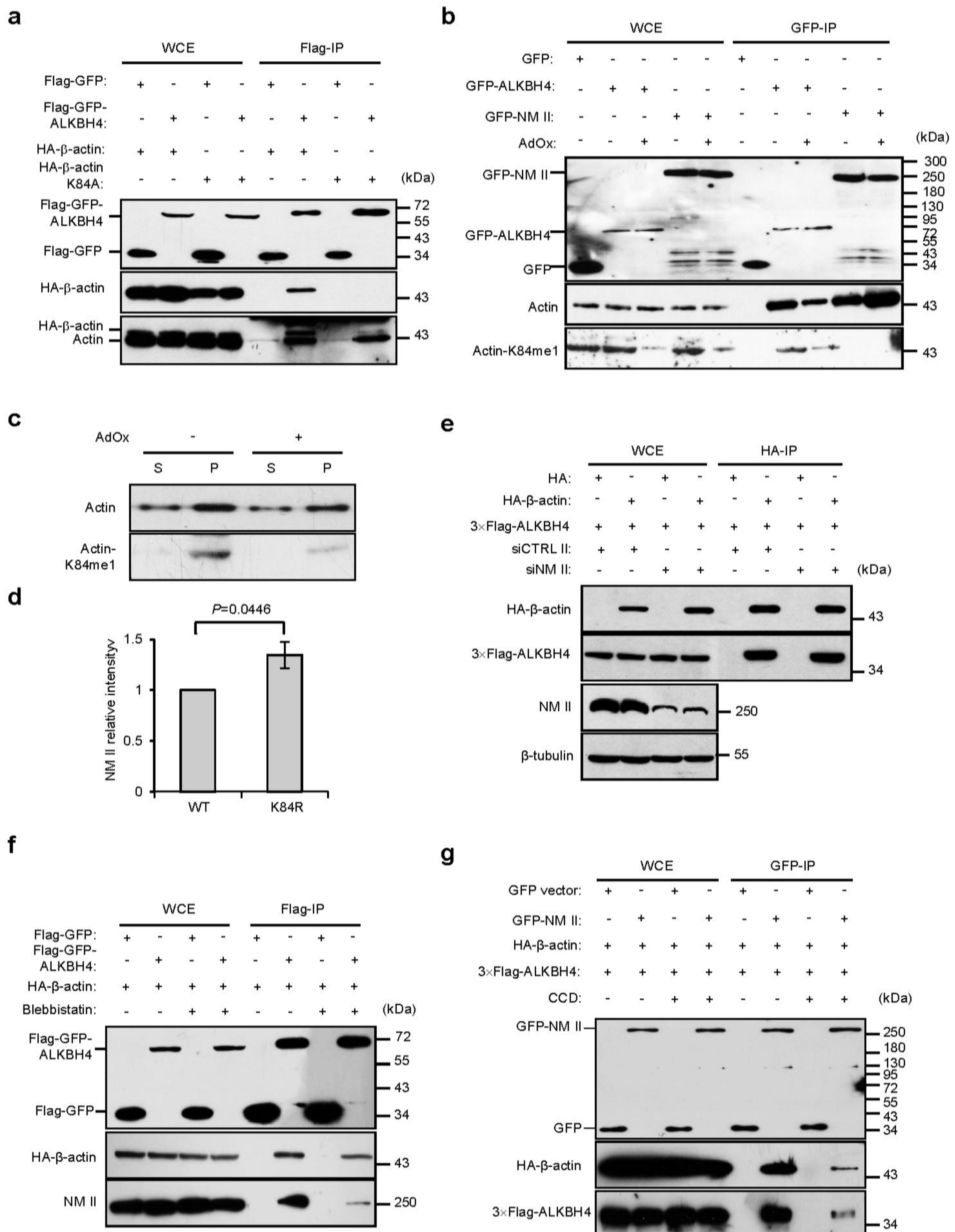
deletion of exons 2 and 3 allele (germline:- or inducible: Δ). Deletion of exon 2 and 3 removes the dioxygenase domain of *Alkbh4* and will invalidate the catalytic domain of both long (AK008083) and short (BC022729) isoforms of Alkbh4. The genomic region of interest was amplified as three fragments from AB2.2 ES cell DNA in the bMQ 337L22 BAC clone (Geneservice). An approx. 4.2 kb sized 5' fragment containing sequences from exon 1 to part of intron 3 was amplified with primers 5'-TTAGAGCCCAAGACTTCCAAGTTCC-3'/ 5'-CAGCACACAGGATAGAAATCCAACC-3'. This subclone was used to generate the 5' small homology arm of the targeting vector. An approx. 3.1 kb sized 3' fragment containing the exons 2 and 3 sequences was amplified with primers 5'-GCTATTGTGGGTCTTTGCTGGTTCC-3'/ 5'-TCTGCACACAGAAACACAAGCCTCC-3'. This fragment was used to generate the proximal part of the 3' long homology arm. An approx. 4.8 kb sized 3' fragment containing part of the exon 3 and downstream sequences was amplified with primers 5'-TTCAGGGAGCTGTCCAGTGAGTTCC-3'/ 5'-TCACTGGAAACTGCTGGAGCCTACC-3'. This subclone was used to generate the distal part of the 3' long homology arm of the targeting vector. The three PCR fragments were subcloned into pCR4-TOPO vector (Invitrogen). A 3510 bp sized SpeI/Bst1107I fragment of the short homology arm was inserted into a G140 vector (Genoway) upstream of the LoxP-FRT-Neomycin-FRT (linearized with AvrII/PmeI). A 3758 bp sized NheI-HpaI fragment from this construct was inserted into a pGA1 vector (Genoway) with inserted polylinker (restriction sites PacI-NruI-XbaI-HpaI-SpeI-AvrII-Bst1107I-NheI-NotI-AscI) linearized with XbaI-HpaI. A synthetic LoxP fragment was inserted into the BglII site downstream exon 3 of the distal part of the distal long homology arm to generate the distal LoxP site. This insertion destroyed the BglII site and introduced PciI and AspI sites. The proximal long arm was joined to the distal long arm by insertion of a 3722bp sized AatII-PmeI fragment isolated from the distal long arm into the proximal long arm cut with AatII-PmlII. A 5887bp sized Bst1107I-SpeI fragment isolated from the long homology arm was inserted into the short homology arm plasmid, linearized with Bst1107I-NheI. The final targeting vector was completed by insertion of a Diphteria Toxin A negative selection cassette at the 3'end long homology arm via AscI-NotI. FRT sites indicated in figure by red ⧖. The targeting vector was linearized with NruI and electroporated into 129Sv/Pas ES cells. ES cells were selected with 200 µg/ml G418. Homologous recombination in ES cells was verified by Southern blot analysis. The Southern blot screening was based on a SpeI and

PciI digestion of genomic DNA and hybridization using an external 524 bp probe (Ext 5'probe) located upstream of the 5' homology sequence and an internal 399 bp probe (Int 3'probe) located within the 3' homology sequence of the targeting vector. Blue- and green- dashed double-end arrow lines representing southern-blotting products using 5'-probe and 3'-probe, to distinguish WT (+) allele from floxed allele (L), respectively. DNA fragments detected by these probes are shown in figure. Ex: exon. **b**, ES cells were injected into C57BL/6N blastocysts and chimeras were bred with C56BL/6 mice to make a germline transmissible *Alkbh4* floxed allele (*Alkbh4^{L}*). Floxed mice were mated with a C56BL/6 Cre-deleter mouse strain (Genoway) to excise the floxed region and generate germline transmissable knock-out allele (-). Mice homozygous for the recombined allele (*Alkbh4$^{L/L}$*) were bred with tamoxifen-inducible Cre-ER mice to generate *Alkbh4$^{L/L}$Cre* mouse strain for the inducible deletion (Δ) of *Alkbh4* in MEF cells. PCR strategy for genotyping: PCR products shown as red lines with corresponding primer sets. Product size shown as indicated. **c**, Representative images of PCR genotyping of *Alkbh4* wild-type (*Alkbh4$^{+/+}$*) and heterozygous conventional/germline knockout mice (*Alkbh4$^{+/-}$*) and Cre-mediated conversion of *Alkbh4* floxed gene (*Alkbh4$^{L/L}$*) to *Alkbh4* deleted (*Alkbh4$^{\Delta/\Delta}$*) obtained from 4-OHT induced *Alkbh4$^{L/L}$Cre* MEF cells. **d**, MRC5 cells were treated with CTRL or ALKBH4 siRNA for 48 h, fixed and immunostained with ALKBH4 antibody (#1) and DAPI. Scale bars, 10 μm. **e**, MRC5 cells were treated with CTRL or ALKBH4 siRNA for 48 h, lysed and analyzed by immunoblotting with the ALKBH4 (#2) and β-tubulin antibodies.



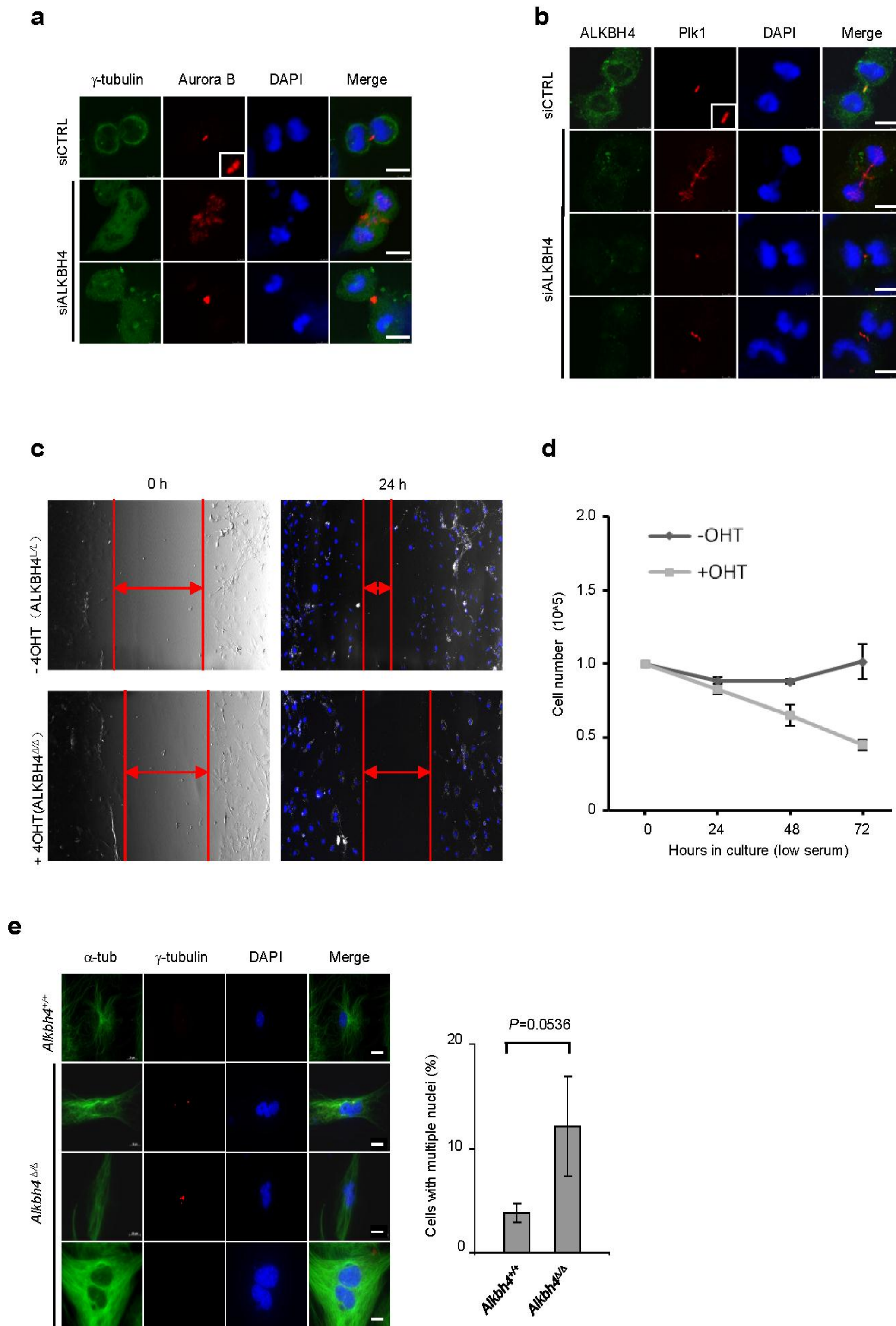
Supplementary Figure S2. Mass spectrometry analysis based screening for ALKBH4 interacting partners and methylated peptide. a, Silver staining of Flag immunoprecipitates from 293T cells transfected with Flag-GFP or Flag-GFP-ALKBH4. NM II and actin were identified in ALKBH4 specific bands by mass spectrometric (MS) analysis. **b**, Specificity of the actin-K84me1 antibody was verified by dot-blotting of non-, mono-, di- and tri-methylated actin peptides synthesized by New England Peptide. **c-d**, High-resolution and high-mass-accuracy fragmentation

spectra of a doubly (**c**) and triply (**d**) charged β-actin peptide that is mono-methylated at K84 are shown. The one originated from the HA-β-actin immunoprecipitated from 293T cells indicated as *in vivo* (upper panel) matched well with that of a synthetic peptide (lower panel).



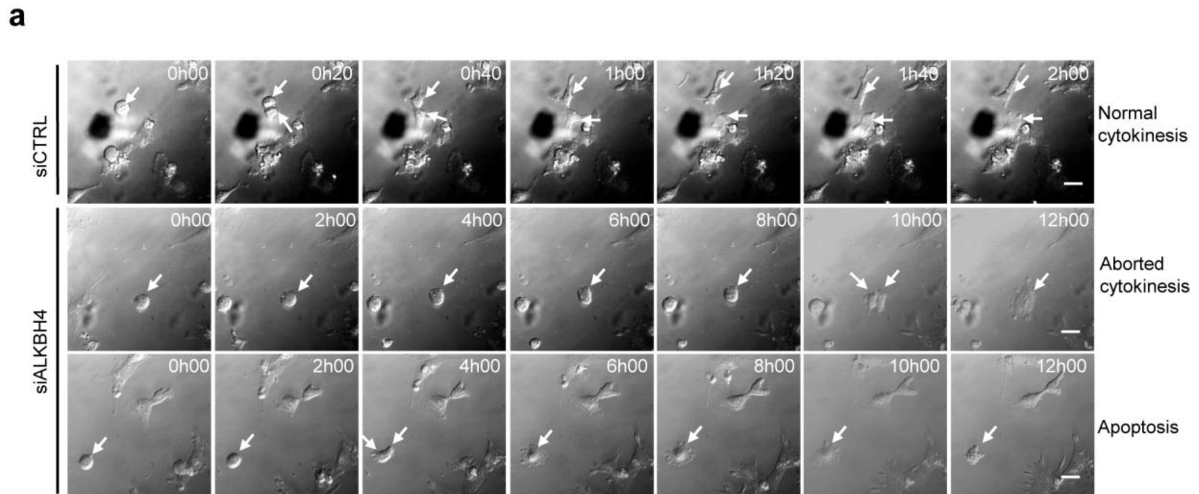
Supplementary Figure S3. Actin mediates the ALKBH4-Actomyosin interaction. a, 293T cells were co-transfected with Flag-GFP-ALKBH4 and HA-β-actin WT or K84A as indicated and lysates were subjected to Flag immunoprecipitation. Immunoprecipitates and WCEs were immunoblotted with the indicated antibodies. **b,** 293T /GFP-NM II or 293T/GFP-ALKBH4 stable cell were treated

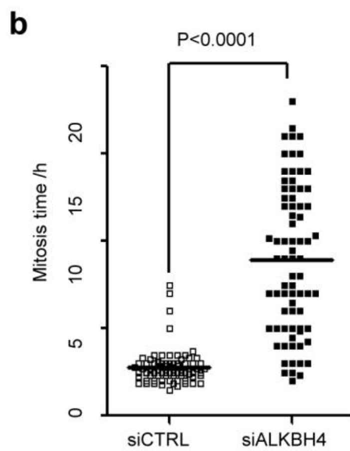
with 20 μM AdOx (Adenosine-2′, 3′-dialdehyde) for 24 h, lysed and subjected to GFP immunoprecipitation. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. **c,** Detection of free and filamentous actin by actin fractionation. 293T cells were treated with 20 μM AdOx for 24 h. Fractions were analyzed by immunoblotting with the indicated antibodies. **d**, Quantification of signal intensity of K84R relative to WT from three independent experiments. 293T cells were transfected with WT or K84R HA-β-actin as indicated and lysates were subjected to HA immunoprecipitation. **e,** 48 h after transfection with the indicated siRNAs and DNA constructs, 293T cells were lysed and subjected to HA immunoprecipitation. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. **f**, 48 h after transfection with the indicated DNA constructs, 293T cells were treated with 5μM Blebbistatin for 5 h, lysed and subjected to Flag immunoprecipitation. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. **g**, 48 h after transfection with the indicated DNA constructs, 293T cells were treated with 1 μg/ml CytochalasinD (CCD) for 1.5 h, lysed and subjected to GFP immunoprecipitation. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

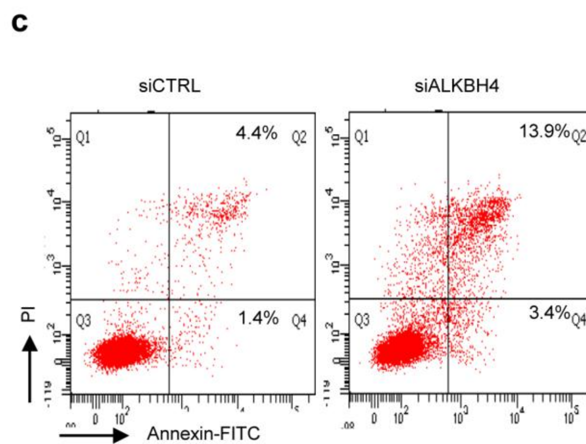


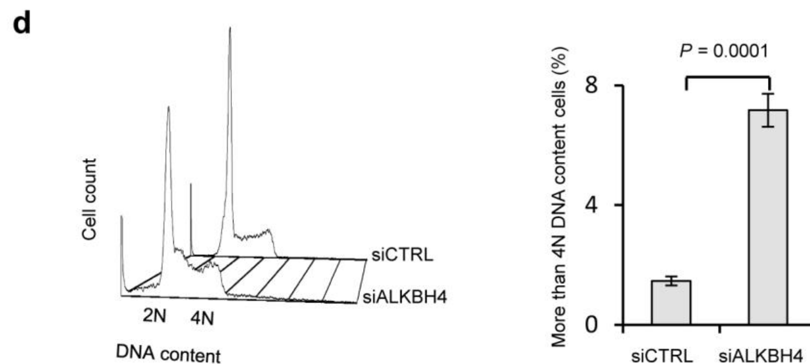
Supplementary Figure S4. Cellular phenotypes associated with ALKBH4 deficiency. a-b, MRC5 cells treated with ALKBH4 or CTRL siRNA were immunostained with γ-tubulin and Aurora B (**a**) or Plk1 and ALKBH4 (**b**). Scale bars, 10 μm. **c**, Representative images of scratch assay with primary *Alkbh4*$^{L/L}$*Cre* MEF cells untreated (*Alkbh4*$^{L/L}$) or treated with 4-OHT (*Alkbh4*$^{Δ/Δ}$). Pictures

were taken at time 0 h when the scratch was made and 24 h later with matched reference points. The images acquired were analyzed by measuring the distances between the borders of the scratches (red arrows). **d**, *Alkbh4*$^{L/L}$*Cre* MEF cells, 4-OHT-treated (*Alkbh4*$^{\Delta/\Delta}$) and untreated (*Alkbh4*$^{L/L}$), show approximately the same amount of cells after 24h in low serum medium, but *Alkbh4*$^{\Delta/\Delta}$ cells start to die off at 48h and on. Cell count of *Alkbh4*$^{L/L}$*Cre* MEF cells, untreated (-OHT) or treated with 4-OHT (+ OHT), replated in low serum medium (0.5%) and harvested at the indicated time points (n = 2, two different primary MEF cell lines). **e**, *Alkbh4*$^{+/+}$*Cre* (*Alkbh4*$^{+/+}$) and *Alkbh4*$^{L/L}$*Cre* (*Alkbh4*$^{\Delta/\Delta}$) MEF cells were treated with 4-OHT and immunostained with the indicated antibodies and DAPI, scale bars 20 µm. Histogram represents the mean of three independent experiments (300 cells/condition/experiment). *P* value was calculated using a two-tailed *t*-test. Error bars indicate s.d.



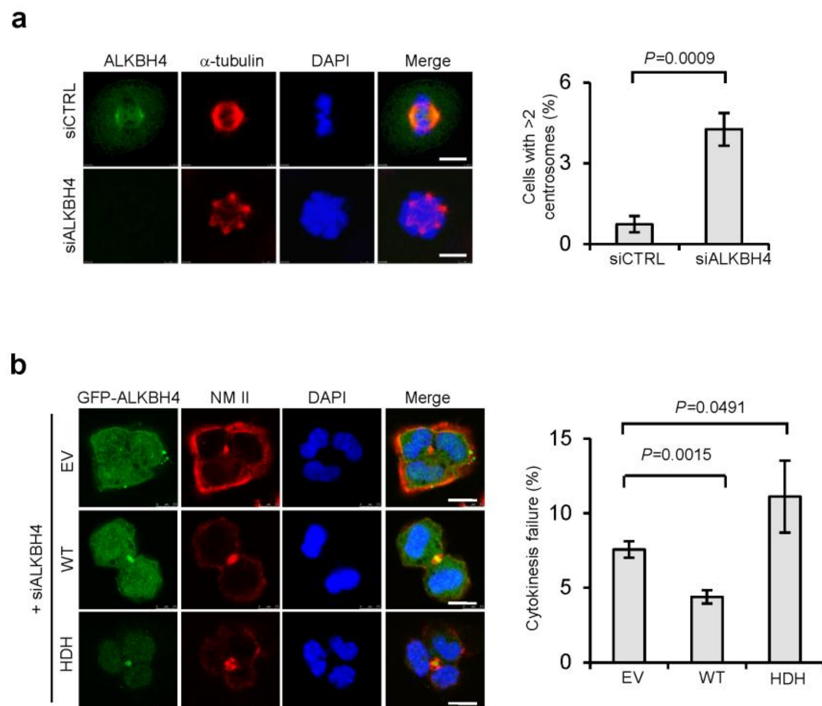




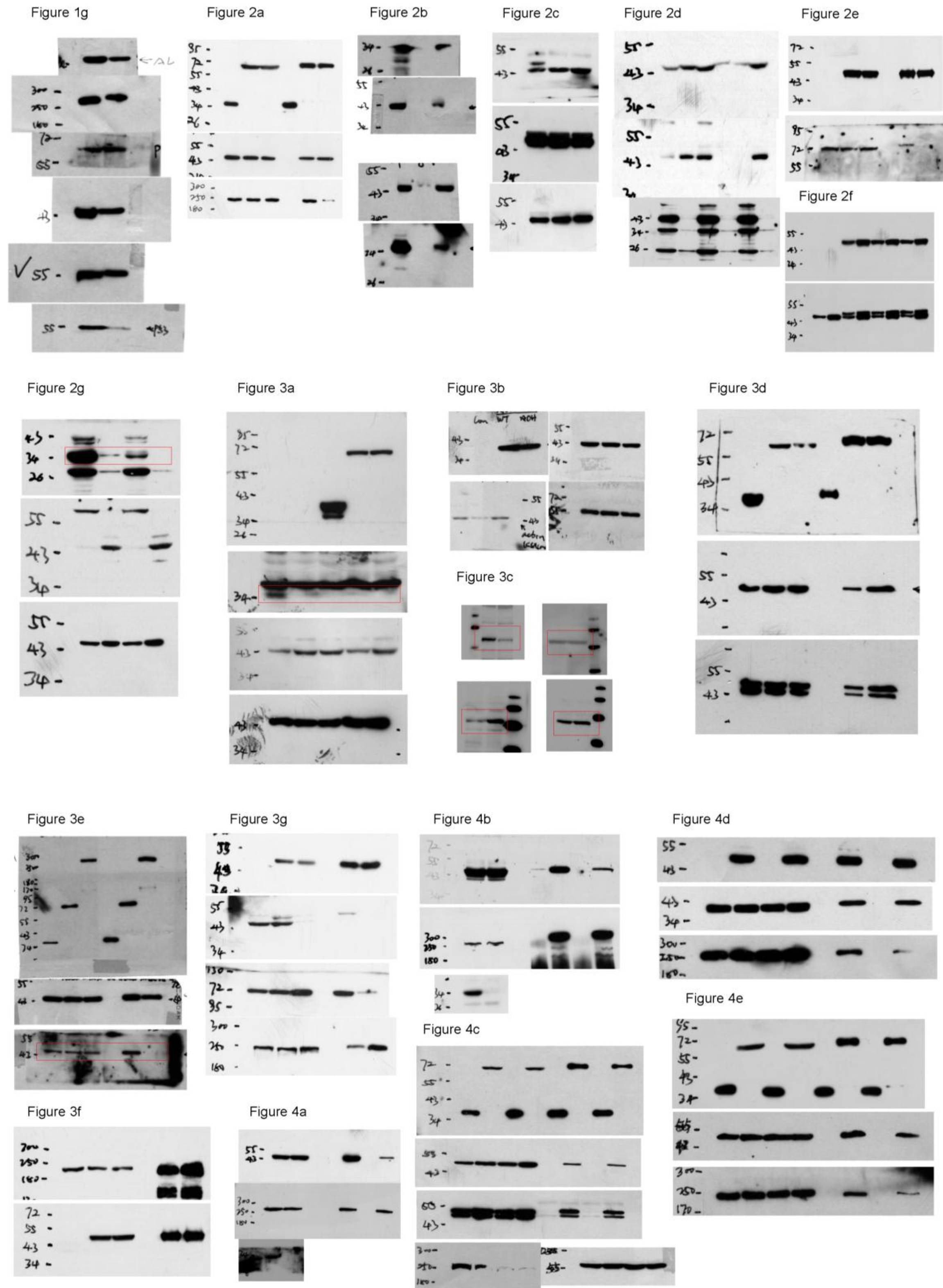


Supplementary Figure S5 ALKBH4 deficiency leads to increased cell death, mitotic index. a, Representative images of successful and failed cytokinesis and apoptosis in MRC5 cells treated with ALKBH4 or CTRL siRNA for 48 h; see Supplementary Movies 1, 2 and 3, respectively. Time is indicated in hours and minutes. Scale bars: 20 μm. **b**, Measurement of live cell mitotic length for ALKBH4 proficient and deficient cells (80 cells/condition/experiment for each genotype). **c**, MRC5 cells were treated with CTRL or ALKBH4 siRNA for 48 h, stained with annexin V and propidium iodide (PI) and analysed by flow cytometry. **d**, MRC5 cells treated with CTRL or ALKBH4 siRNA

for 48 h were stained with propidium iodide (PI) and analysed by flow cytometry. Quantification of > 4N DNA content cells is based on three independent experiments (30,000 cells/condition/experiment).



Supplementary Figure S6. ALKBH4 deficiency leads to centrosome supernumerary and cytokinesis failure. a, MRC5 cells treated with ALKBH4 or CTRL siRNA were immunostained with α-tubulin and ALKBH4. Graph represents the mean of three independent experiments (300 cells/condition/experiment). *P* value was calculated using a two-tailed *t*-test. Error bars indicate s.d. Scale bars, 10 µm. **b**, MRC5 cells were simultaneously transfected with ALKBH4 siRNA and the indicated siRNA insensitive Flag-GFP-ALKBH4 constructs for 48 h, fixed and immunostained with the NM II antibody and DAPI. EV, empty vector. Quantification was based on three independent experiments (100 cells/condition/experiment). *P* value was calculated using a two-tailed *t*-test. Error bars indicate s.d. Scale bars, 10 µm.



Supplementary Figure S7. Full scans of the key immunoblots.

Supplementary Table S1. A full list of all identified proteins from ALKBH4 pull down

Accession #	Coverage	Peptides	#AAs	MW [Da]	Score	Description
IPI:IPI00033770.5	**66.23**	**42**	**302**	**33816**	**149.50**	**Isoform 1 of Alkylated DNA repair protein alkB homolog 4**
IPI:IPI00220327.3	36.34	35	644	65978	123.11	Keratin, type II cytoskeletal 1
IPI:IPI00304925.5	35.88	26	641	70009	95.03	Heat shock 70 kDa protein 1
IPI:IPI00009865.2	29.01	28	593	59475	94.77	Keratin, type I cytoskeletal 10
IPI:IPI00021439.1	**50.13**	**23**	**375**	**41710**	**76.43**	**Actin, cytoplasmic 1**
IPI:IPI00021304.1	17.21	16	645	65825	54.12	Keratin, type II cytoskeletal 2 epidermal
IPI:IPI00514530.5	29.76	14	289	32254	43.89	Putative uncharacterized protein ACTA1
IPI:IPI00396485.3	14.72	11	462	50109	38.53	Elongation factor 1-alpha 1
IPI:IPI00019359.3	17.82	11	623	62092	35.95	Keratin, type I cytoskeletal 9
IPI:IPI00796776.1	11.11	8	567	60089	25.56	cDNA FLJ54081, highly similar to Keratin, type II cytoskeletal 5
IPI:IPI00554648.3	6.21	5	483	53671	17.99	Keratin, type II cytoskeletal 8
IPI:IPI00384444.5	5.72	5	472	51589	17.43	Keratin, type I cytoskeletal 14
IPI:IPI00465248.5	9.91	3	434	47139	9.34	Isoform alpha-enolase of Alpha-enolase
IPI:IPI00908770.1	8.20	2	317	35897	7.55	cDNA FLJ53063, highly similar to Tubulin beta-7 chain
IPI:IPI00074893.1	2.13	2	892	97202	7.28	Zinc finger protein 512B
IPI:IPI00007856.1	**1.03**	**1**	**1941**	**222906**	**6.52**	**Myosin-2**
IPI:IPI00647809.1	5.42	3	295	32624	6.50	Chromosome 9 open reading frame 156
IPI:IPI00888126.1	6.59	2	364	39567	6.49	similar to pyruvate kinase, muscle
IPI:IPI00028091.3	2.39	2	418	47341	6.29	Actin-related protein 3
IPI:IPI00218161.1	4.68	2	449	51221	5.67	Isoform 3 of High affinity cAMP-specific and IBMX-insensitive 3',5'-cyclic phosphodiesterase 8A
IPI:IPI00641244.1	11.34	2	97	10670	5.50	11 kDa protein
IPI:IPI00387111.5	12.26	2	106	11490	5.37	Uncharacterized protein ENSP00000333154
IPI:IPI00794605.1	7.45	1	161	17596	4.89	18 kDa protein
IPI:IPI00027547.2	12.73	1	110	11277	4.58	Dermcidin
IPI:IPI00867509.1	2.09	1	527	58910	4.34	Coronin-1C_i3 protein

IPI:IPI00641825.2	33.33	1	30	3640	3.96	Phospholipase C, beta 1
IPI:IPI00166202.3	3.06	1	589	65478	3.73	C-type lectin, superfamily member 13
IPI:IPI00873681.2	2.03	1	493	56946	3.66	57 kDa protein
IPI:IPI00305289.2	1.23	1	1056	119085	2.76	Kinesin-like protein KIF11
IPI:IPI00645641.2	3.83	1	784	87525	2.59	cDNA FLJ58439
IPI:IPI00410582.3	3.74	1	294	34172	2.54	Isoform Gamma of Tripartite motif-containing protein 4
IPI:IPI00470589.2	0.83	1	1560	166617	2.48	Isoform 2 of NHR domain-containing protein KIAA1787
IPI:IPI00742780.1	1.78	1	563	65730	2.19	FLJ00279 protein (Fragment)
IPI:IPI00908896.1	6.29	1	159	16839	2.17	cDNA FLJ54533, highly similar to Heterogeneous nuclear ribonucleoprotein H
IPI:IPI00413955.2	1.75	1	744	81740	2.14	cDNA FLJ11853 fis, clone HEMBA1006758, highly similar to Homo sapiens protocadherin beta 13 (PCDH-beta13) mRNA
IPI:IPI00910316.1	1.56	1	514	56646	2.01	cDNA FLJ54333, highly similar to T-complex protein 1 subunit epsilon

Supplementary Table S2. β-actin K84 mono-methylation was identified with a total of 26 spectra of +2, +3, or +4 charge states from a HA-β-actin immunoprecipitated sample indicated as *in vivo*. Only high-confidence search results are shown after filtering.

Sequence	Xcorr	DeltCN	Confident %	Obs[M+H+]	Calc[M+H+]	Δm(PPM)	SpR	Probility Score	Ion % Matched	Spectra count	Charge State
D.DMEK(14.0157)IWHHTFYNELR.V	4.9339	0.4761	100.00%	2032.979	2032.9698	4.5	1	7.71	65.20%	13	3
D.DMEK(14.0157)IWHHTFYNELR.V	3.8567	0.3748	99.20%	2033.9806	2032.9698	3.6	1	5.4	36.40%	4	4
D.DMEK(14.0157)IWHHTFYNELR.V	5.9979	0.3611	98.30%	2034.9766	2032.9698	0	1	6.27	71.40%	9	2