Somatic Mutations in the Angiopoietin-Receptor TIE2 Can Cause Both Solitary and Multiple Sporadic Venous Malformations

Nisha Limaye[1*], Vinciane Wouters[1*], Melanie Uebelhoer[1#], Marjut Tuominen[2#], Riikka Wirkkala[2], John B. Mulliken[3], Lauri Eklund[2], Laurence M. Boon[1,4], and Miikka Vikkula[1]

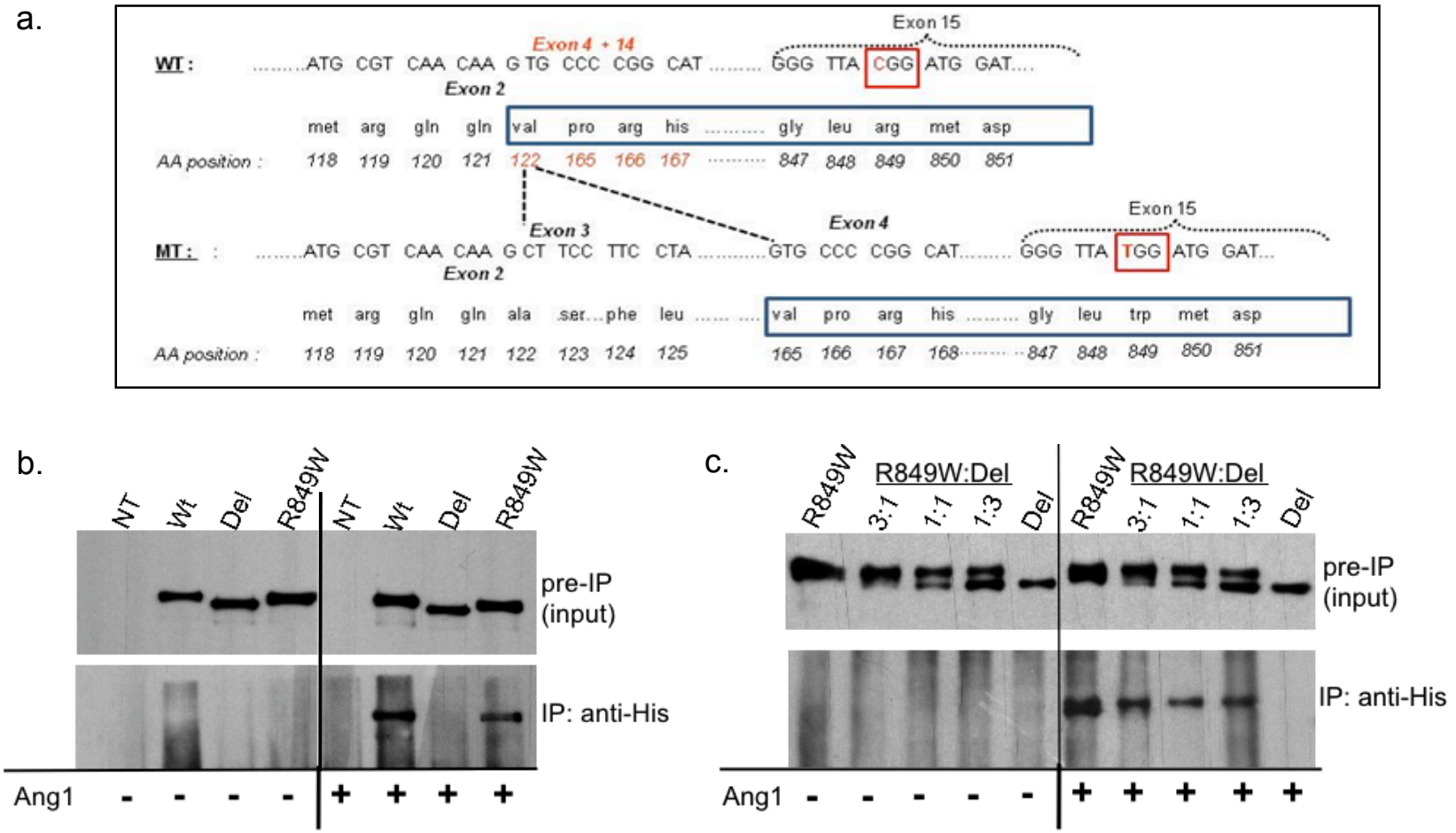
[1] de Duve Institute, Université catholique de Louvain, Brussels, Belgium; [2]Oulu Center for Cell-Matrix Research, Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, University of Oulu, Finland; [3] Department of Plastic Surgery, Children's Hospital and Harvard Medical School, Boston, MA, USA; [4] Center for Vascular Anomalies, Division of Plastic Surgery, Cliniques Universitaires St-Luc, Université catholique de Louvain, Brussels, Belgium.

* and # : These authors contributed equally to this work.

Corresponding author's name:

Pr. Miikka Vikkula, Laboratory of Human Molecular Genetics, de Duve Institute, Université catholique de Louvain, Ave. Hippocrate 74, B-1200, Brussels, Belgium.

e-mail : miikka.vikkula@uclouvain.be

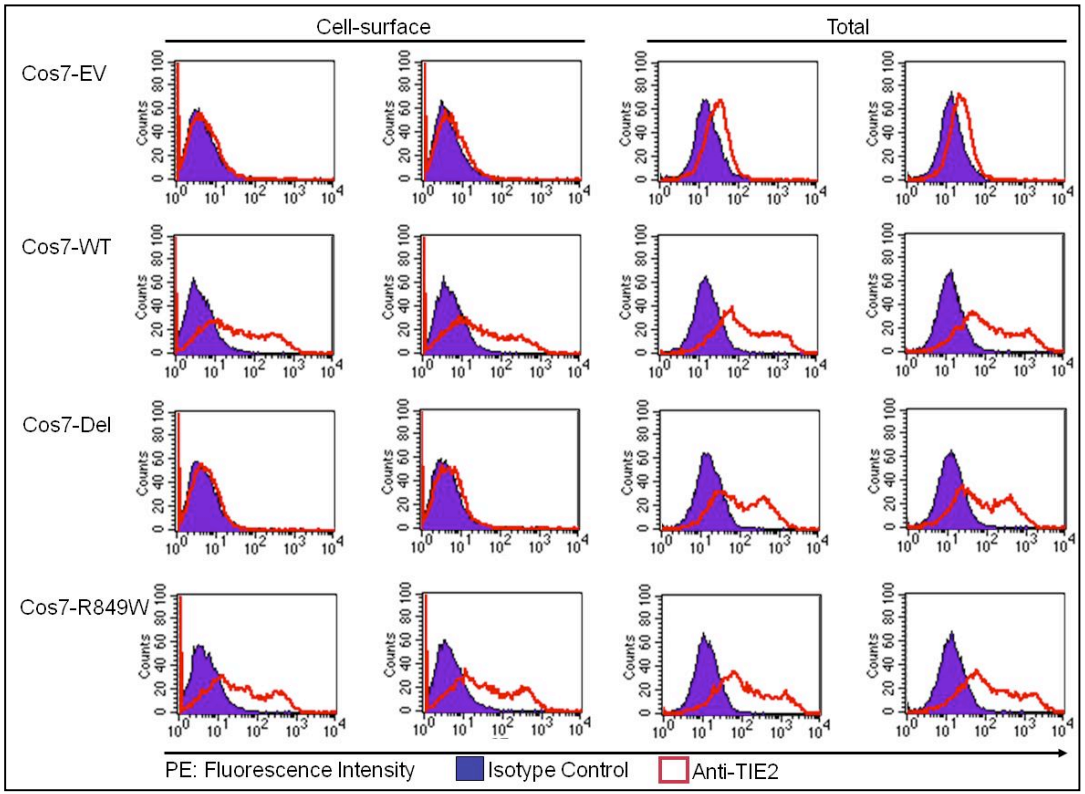


Supplementary Figure 1: Somatic deletion of part of TIE2 *Ig2* in a VMCM.

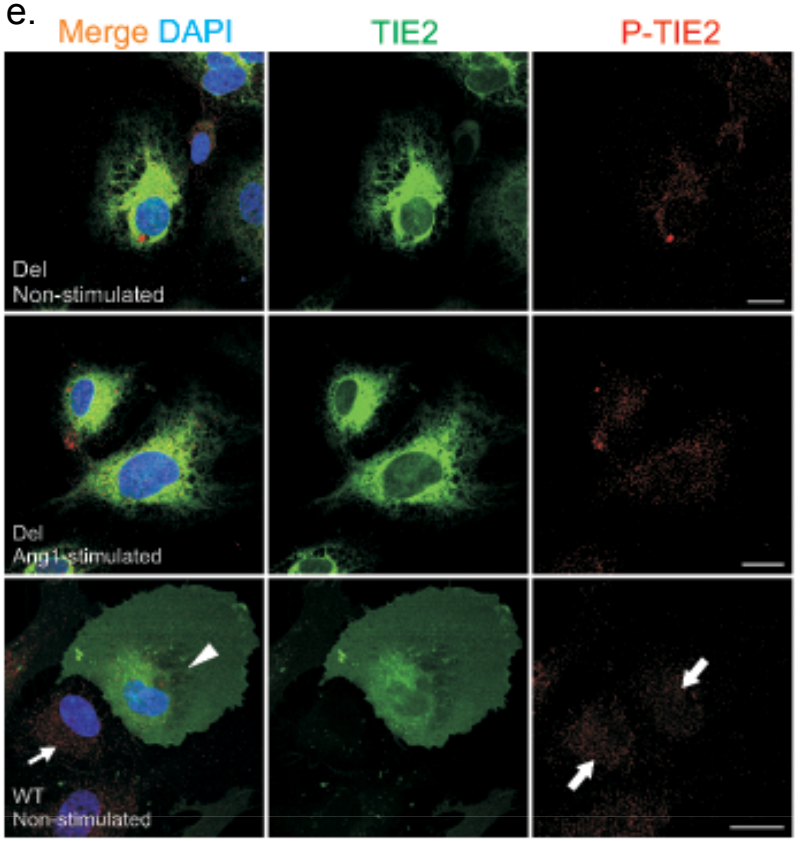
(a) Nucleotide and amino acid sequence-alignments of WT and R849W (MT) alleles in Sa-I.4 lesion. Somatic deletion of exon 3 and first 14 nucleotides of exon 4 occurs on WT allele (CGG i.e. R849 in exon 15), and results in loss of 43 amino acids (A122 to S164) within *Ig2* ligand-binding domain. MT (TGG, i.e. 849W) does not contain this deletion.

(b-c) Western blots for TIE2, on lysates from Cos-7 cells transfected with **(b)** WT, R849W, or Del-TIE2, or **(c)** various ratios of R849W:Del mutants. NT: Non-transfected controls. Cells incubated with ("Ang1 +" lanes) or without ("Ang1 -" lanes) His-tagged Ang1, and immunoprecipitated using anti-His antibody, to assess for ligand-binding. Pre-IP input whole cell lysates in upper panels, show robust TIE2 expression of all expressed forms; 43 amino-acid size difference allows for discrimination between co-expressed R849W and Del-TIE2. Bead-bound immunoprecipitates in lower panels show TIE2 co-immunoprecipitated by anti-His antibody only when bound to His-Ang1 (Ang1 "+" compared to Ang1 "-" lanes), with exception of Del, which fails to co-immunoprecipitate with His-Ang1 (**b**, Ang1 "+" lane 3, and **c**. Ang1 "+" lane 5, absence of lower [Del], lanes 1-4).

d.



e.



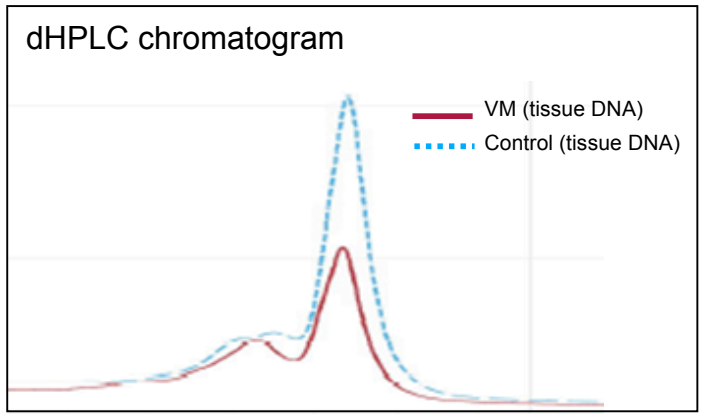
Supplementary Figure 1: Somatic deletion of part of TIE2 *Ig2* in a VMCM.

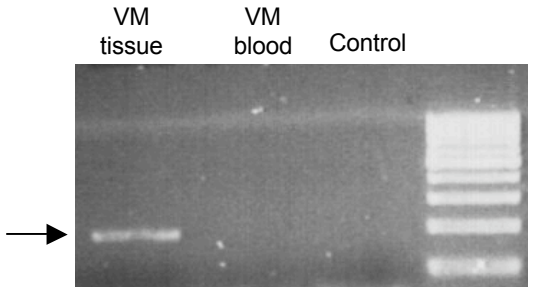
(d) Flow cytometry histograms showing cell-surface (left panels) and total (intracellular +cell surface; right panels) TIE2 staining, carried out on duplicate transfections of EV, WT, R849W, or Del in Cos-7 cells.

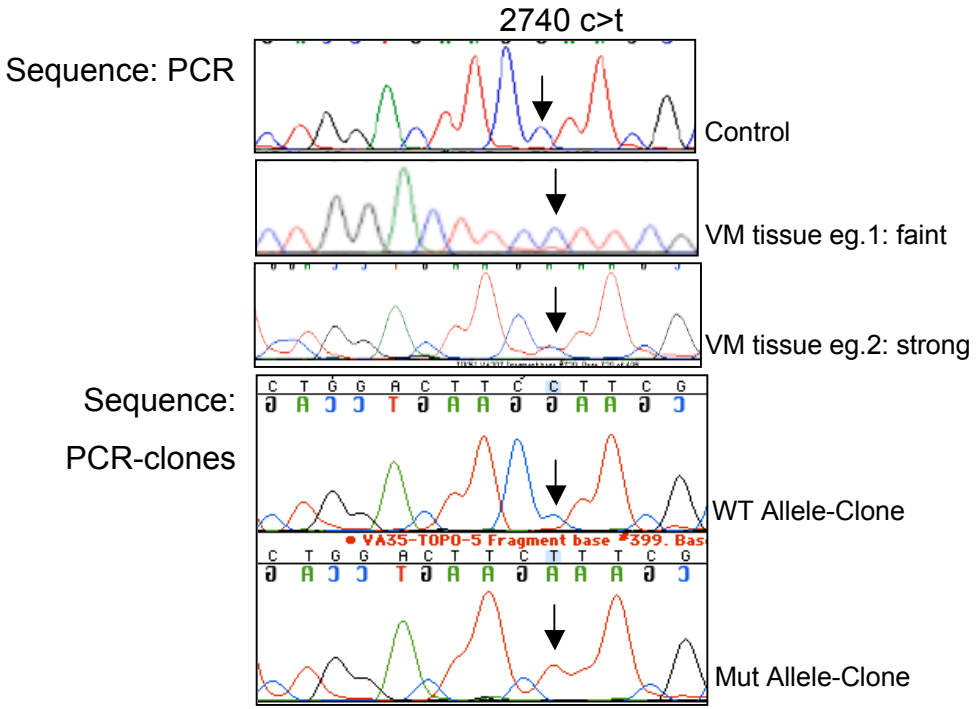
(e) Del-TIE2 accumulated in ER, and not clustered or activated by Ang1. Sparse HUVECs expressing TIE2-Del stimulated for one hour with Ang1 or left unstimulated, and stained with antibodies against total TIE2 (TIE2) and phosphorylated TIE2 (P-TIE2) (top & middle panels). TIE2 remained accumulated in perinuclear area, and did not show any increase in phosphorylation with Ang1-treatment. Same background staining (thick arrows) observed in WT-TIE2 low (small arrow) and high (arrowhead) expressing HUVECs, indicating that P-TIE2 antibody does not cross-react with unphosphorylated TIE2 (bottom panel). Scale bar 20 µm.

Supplementary Figure 2. Screening and identification of TIE2 mutations in VMs

a. L914F: 2740 c>t

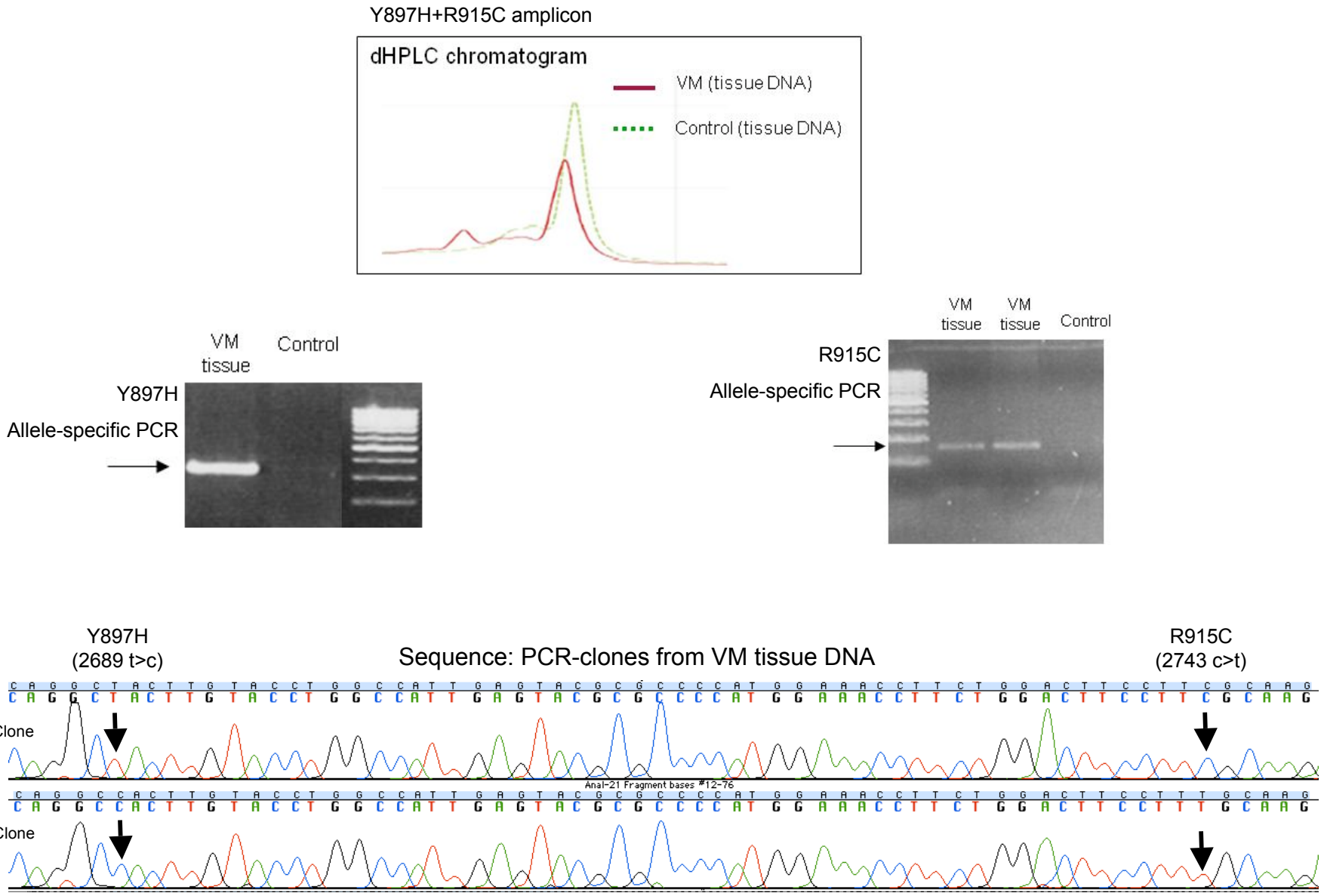






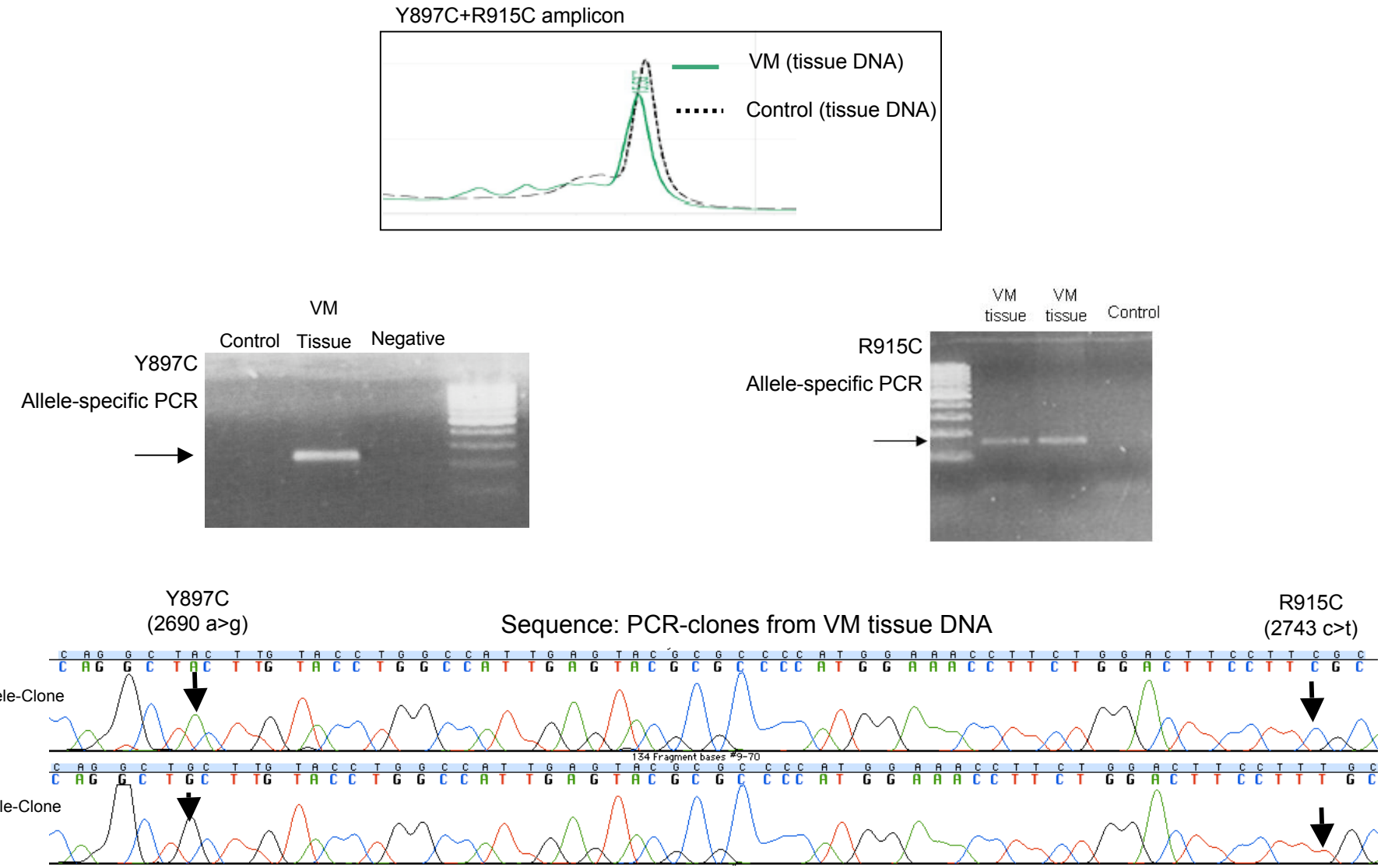
Supplementary Figure 2. Screening and identification of TIE2 mutations in VMs

b. Y897H (2689 t>c)+ R915C (2743 c>t)



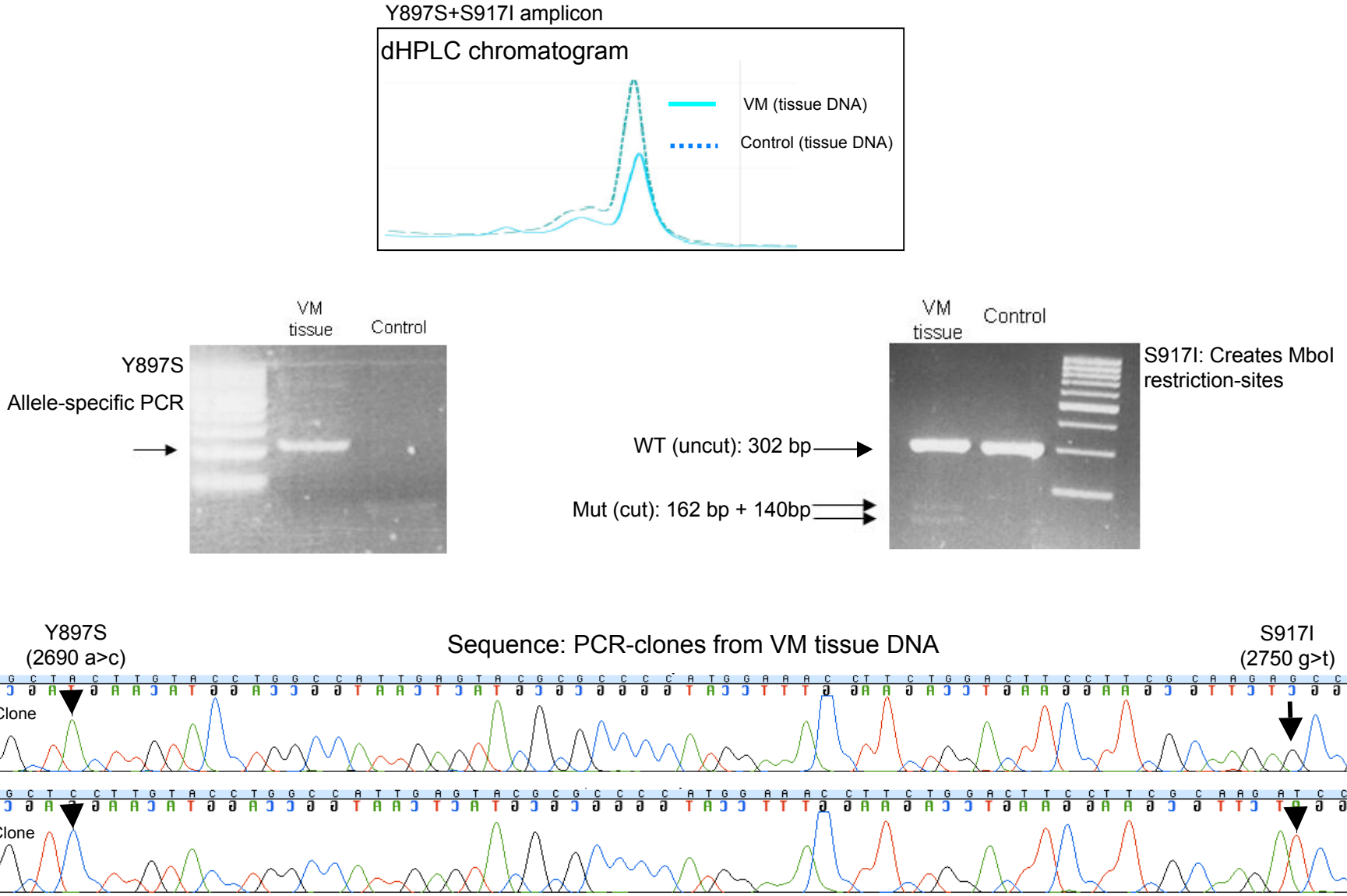
Supplementary Figure 2. Screening and identification of TIE2 mutations in VMs

c. Y897C (2690 a>g)+ R915C (2743 c>t)



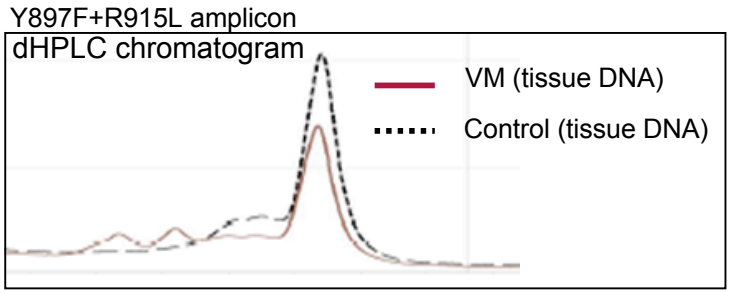
Supplementary Figure 2. Screening and identification of TIE2 mutations in VMs

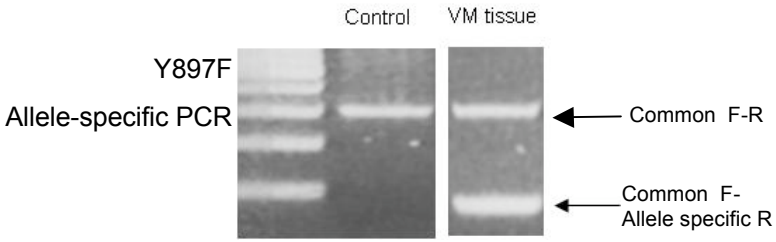
d. Y897S (2690 a>c)+ S917I (2750 g>t)

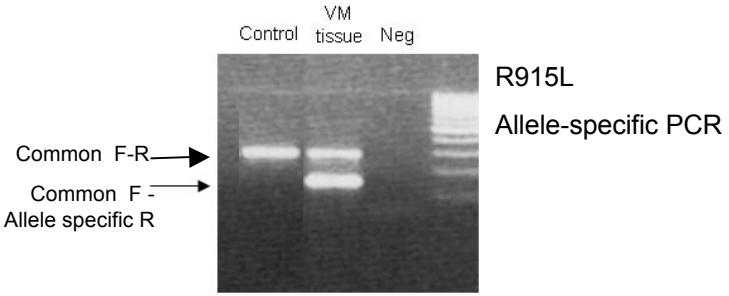


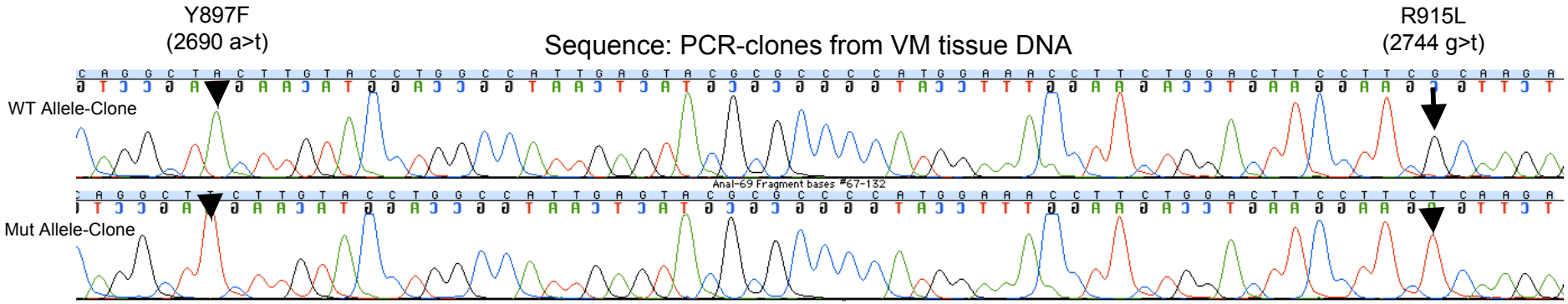
Supplementary Figure 2. Screening and identification of TIE2 mutations in VMs

e. Y897F (2690 a>t)+ R915L (2744 g>t)







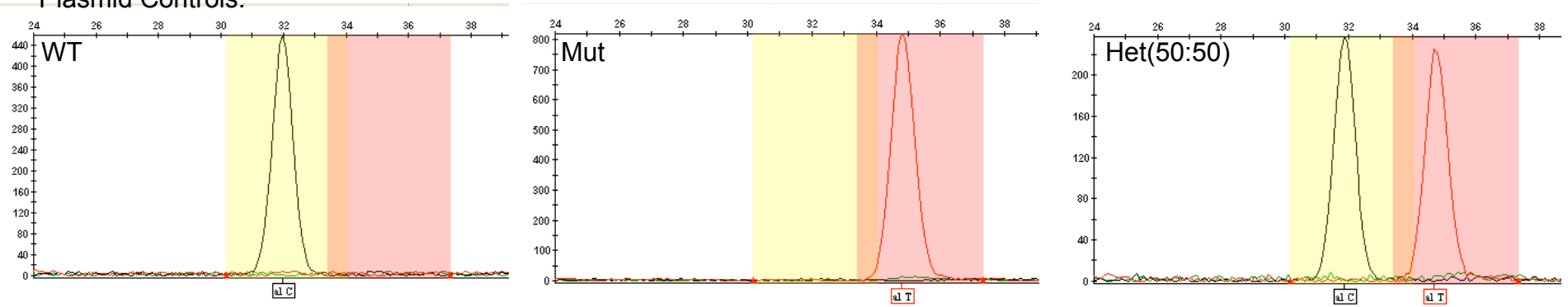


Supplementary Figure 2. (a-e) Screening and identification of TIE2 mutations in VMs. (a) L914F, **(b-e)** TIE2 double *cis*-mutations. dHPLC chromatograms show abnormal elution patterns of exon 17 amplicons from samples containing TIE2 mutations, as compared to controls. Agarose gels show allele-specific PCRs or restriction-digests (S917I) used to discriminate between wild-type and mutant alleles in each case. Most allele-specific PCRs combined a common primer with an allele-specific primer, resulting in one band; where two bands are observed (Y897F and R915L), PCR was carried out using a common forward and reverse, competing with a third allele-specific primer: the common and allele-specific products are discriminated by size. Arrows indicate position of positive (mutant-specific) bands. Sequence data show nucleotide changes identified (indicated above chromatograms, arrows indicate mutated-peak position). For frequent L914F change, PCR-product sequence from tissues with high (weak mutant-signal) and low (strong mutant-signal) levels of tissue heterogeneity shown, along with sequences of wild-type *vs.* mutant PCR-clones. Sequences of PCR-clones from tissues carrying each double-mutant combination, show them occurring in *cis* (on the same allele).

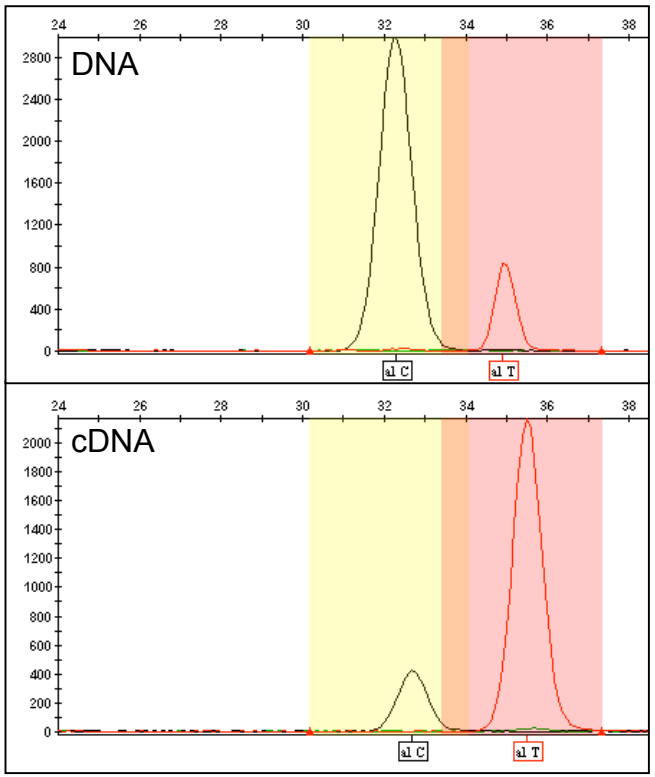
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

a. SNaPshot Assay, L914F Forward: c>t

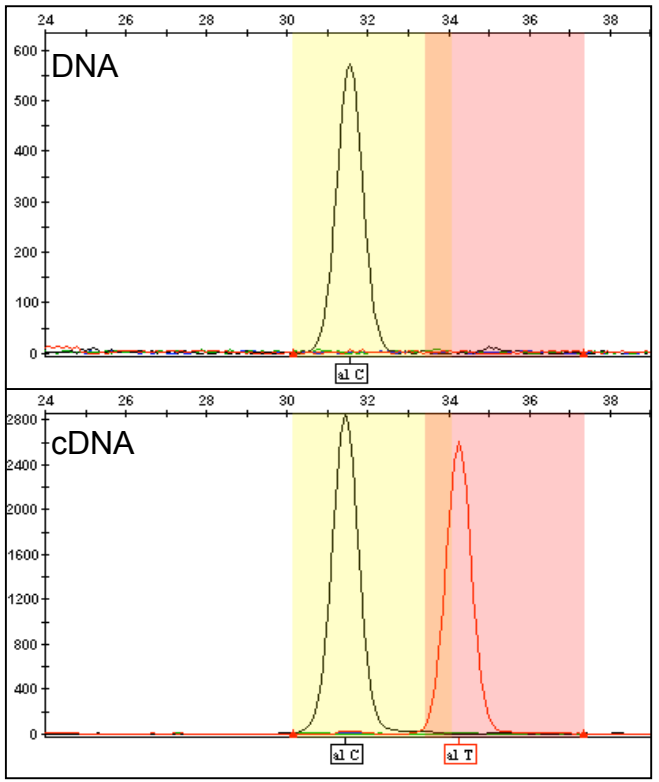
Plasmid Controls:



VM Tissue (4)



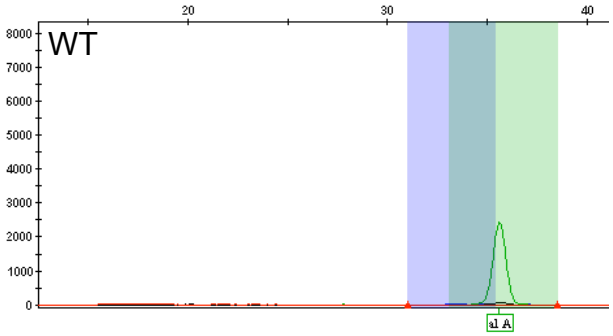
VM Tissue (6)

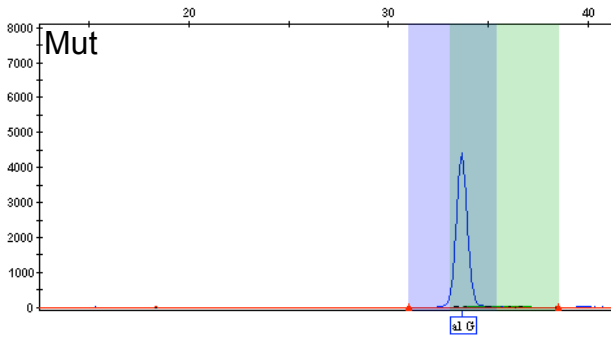


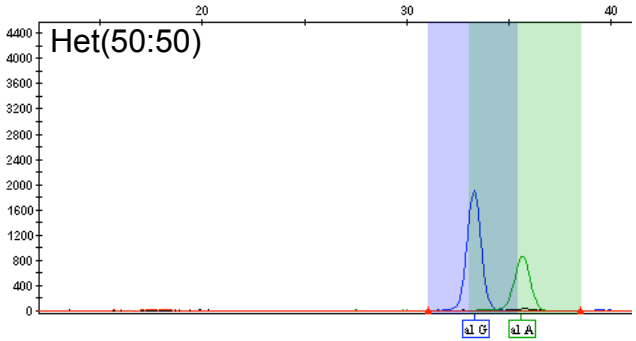
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

b. SNaPshot Assay, Y897H Reverse: a>g

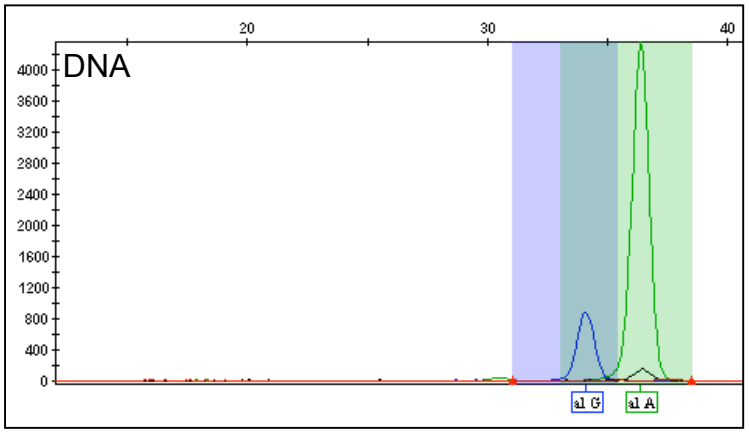
Plasmid Controls:

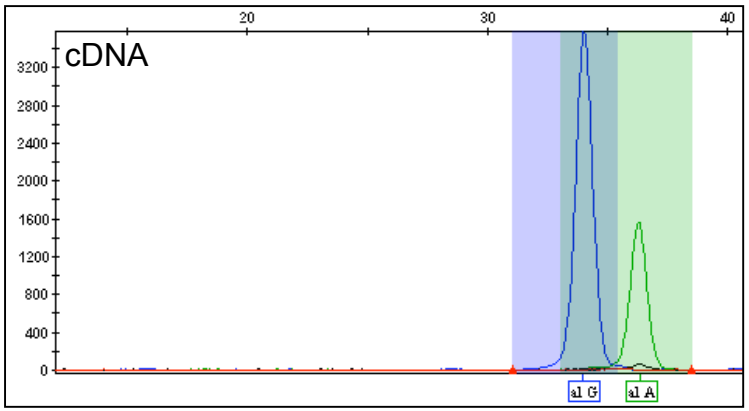






VM Tissue (25)

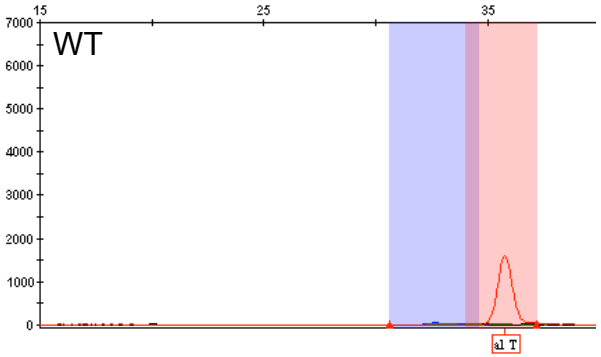


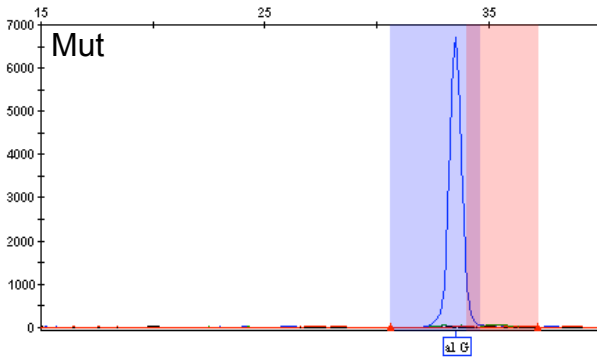


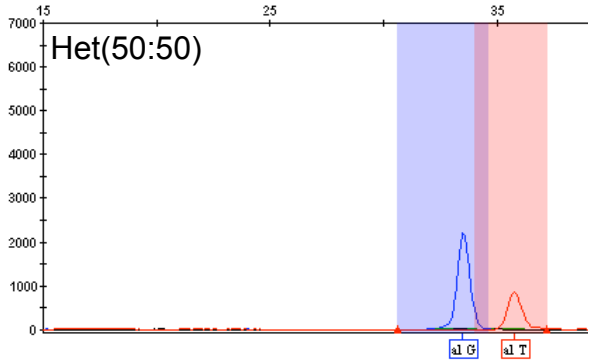
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

c. SNaPshot Assay, Y897S Reverse: t>g

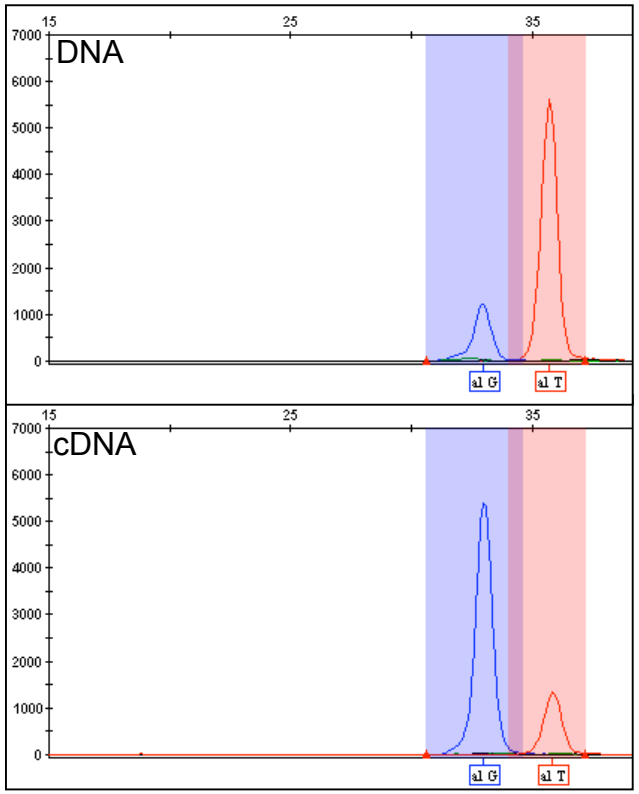
Plasmid Controls:







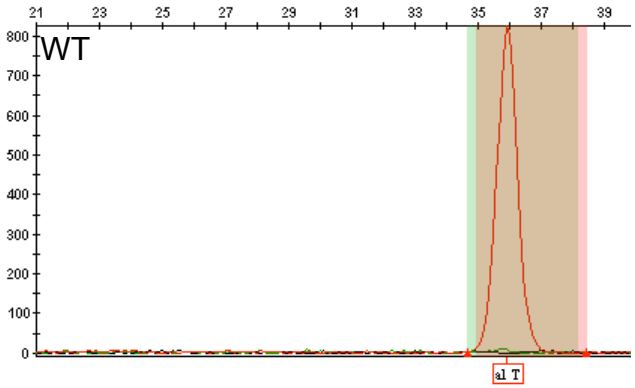
VM Tissue (26)

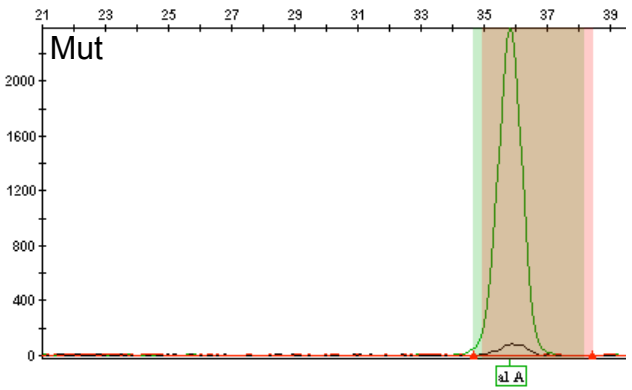


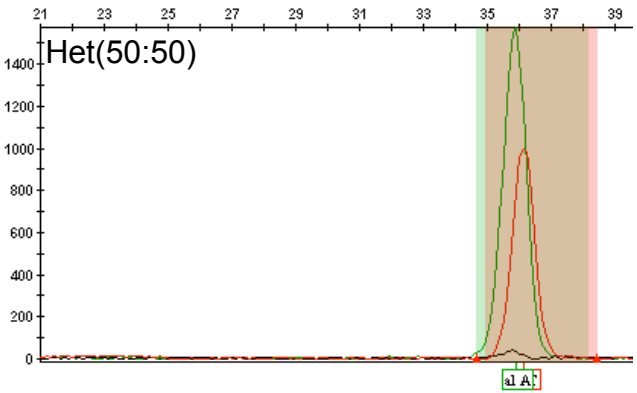
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

d. SNaPshot Assay, Y897F Reverse: t>a

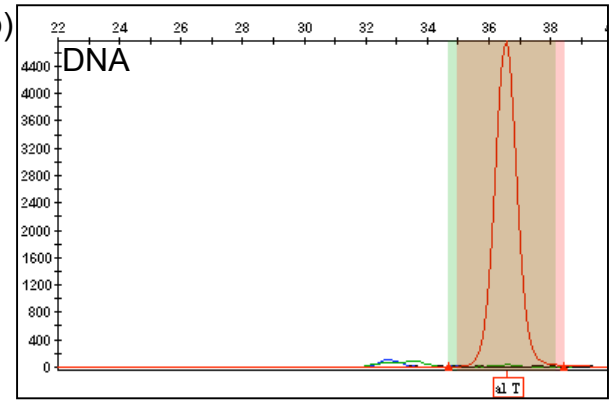
Plasmid Controls:

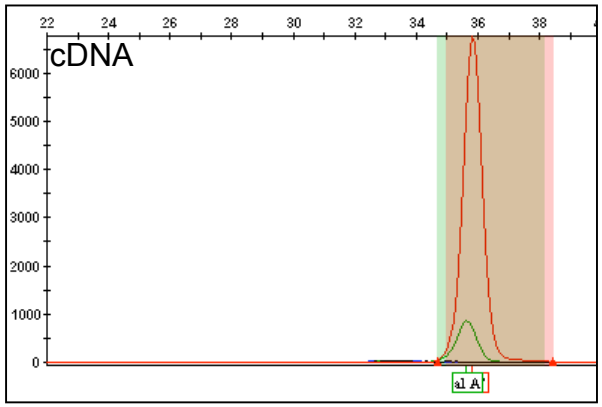






VM Tissue (27b)

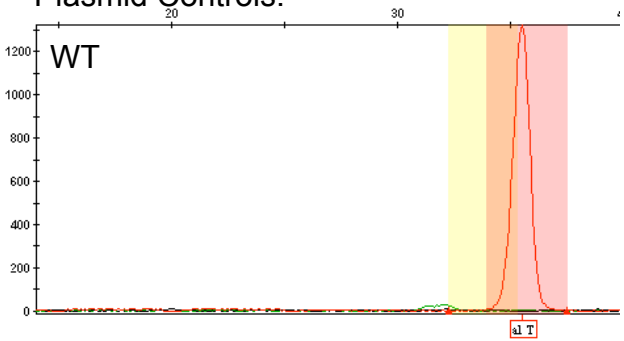


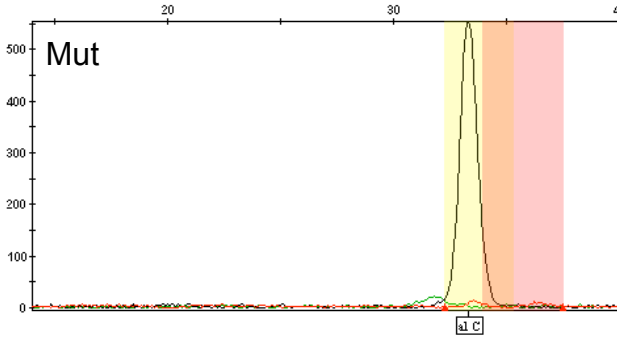


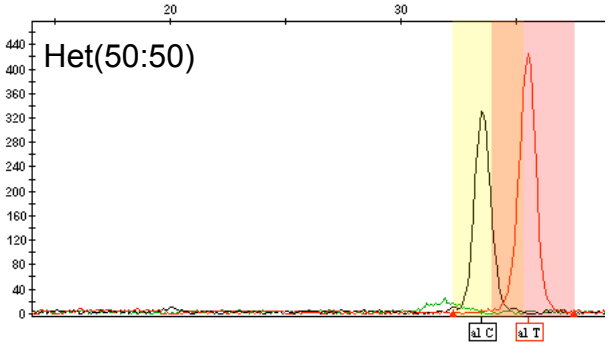
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

e. SNaPshot Assay, Y897C Reverse: t>c

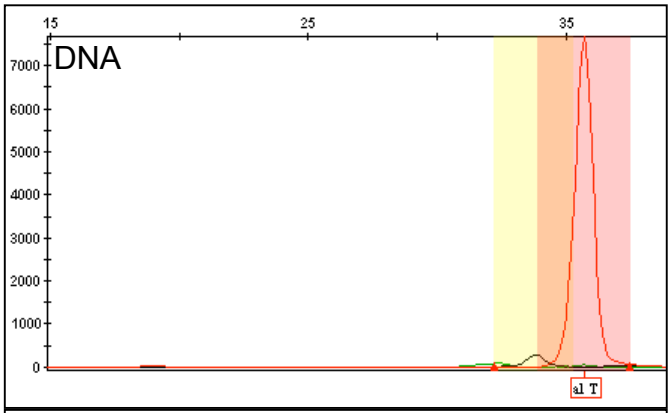
Plasmid Controls:

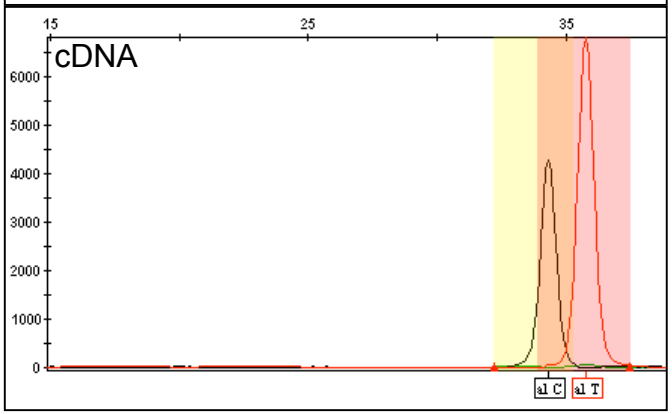






VM Tissue (28b)

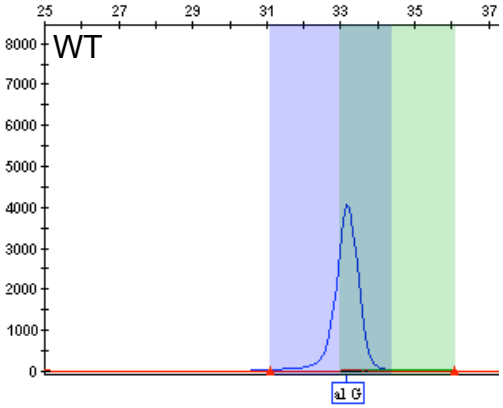


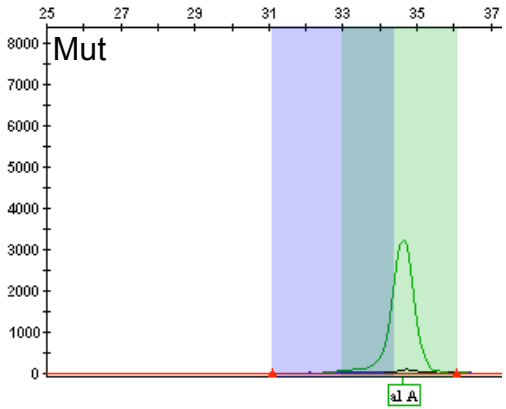


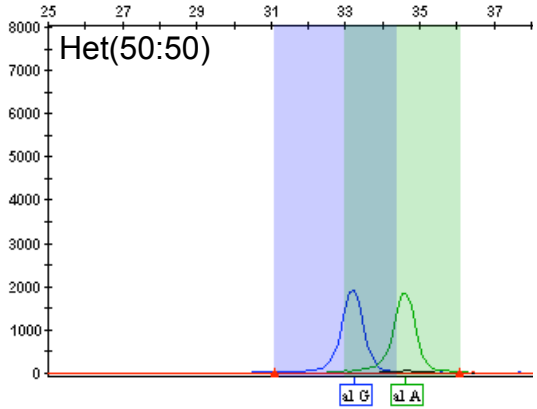
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

f. SNaPshot Assay, R915C Reverse: g>a

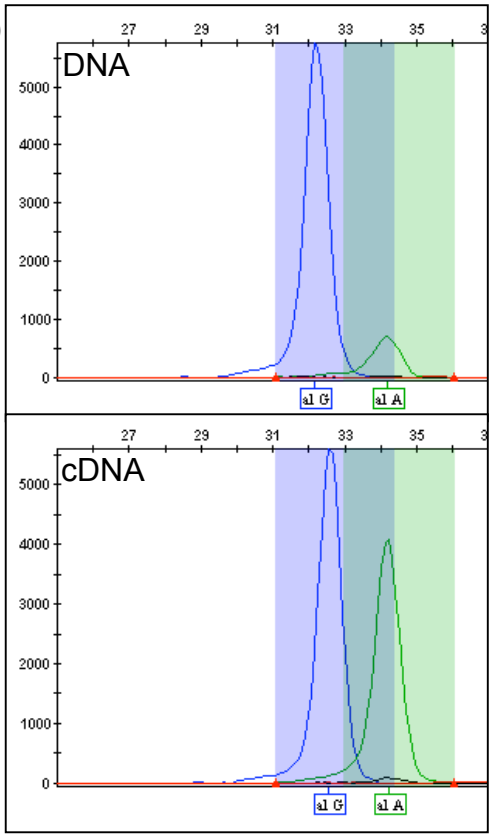
Plasmid Controls:







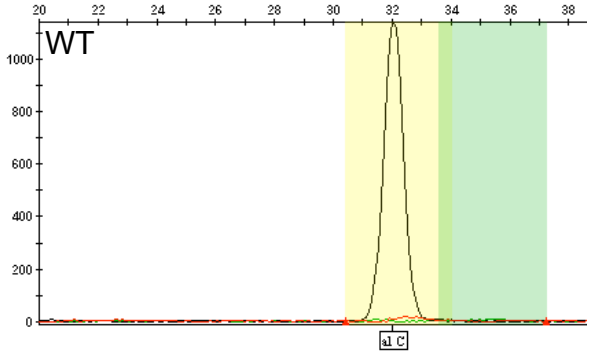
VM Tissue (28b)

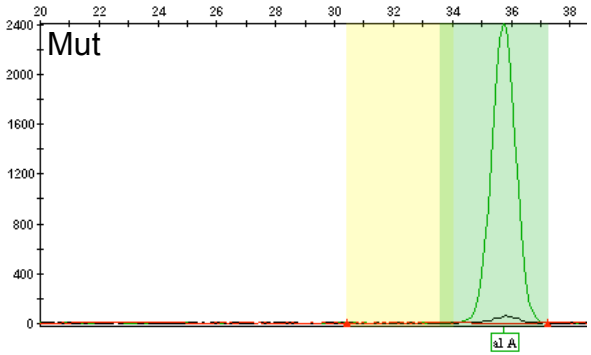


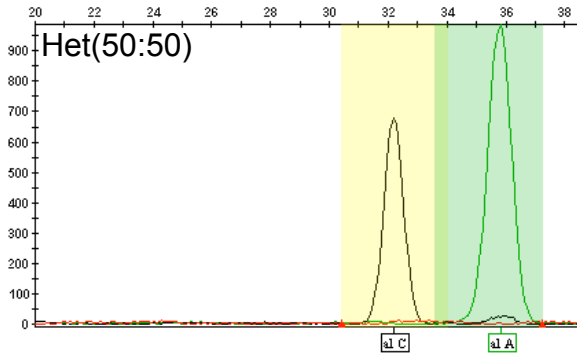
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

g. SNaPshot Assay, R915L Reverse: c>a

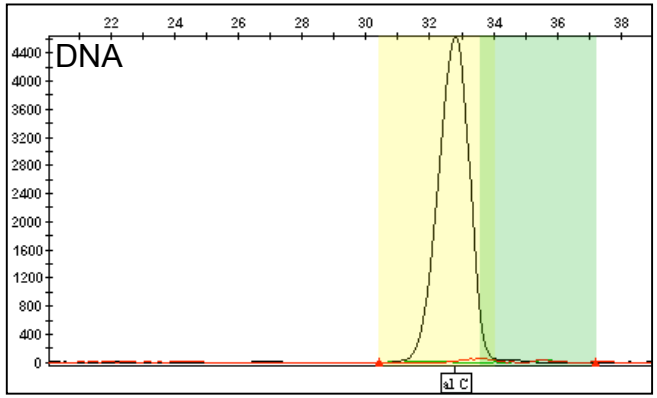
Plasmid Controls:

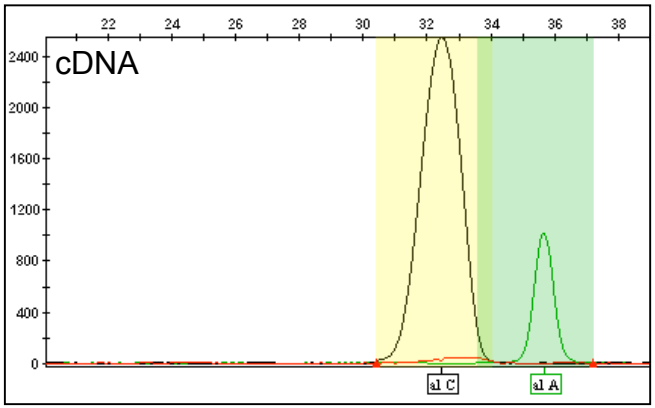






VM Tissue (27b)

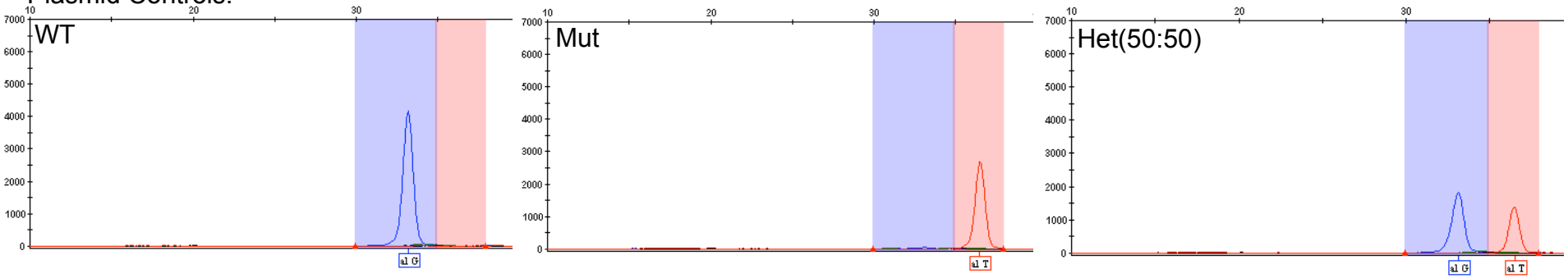




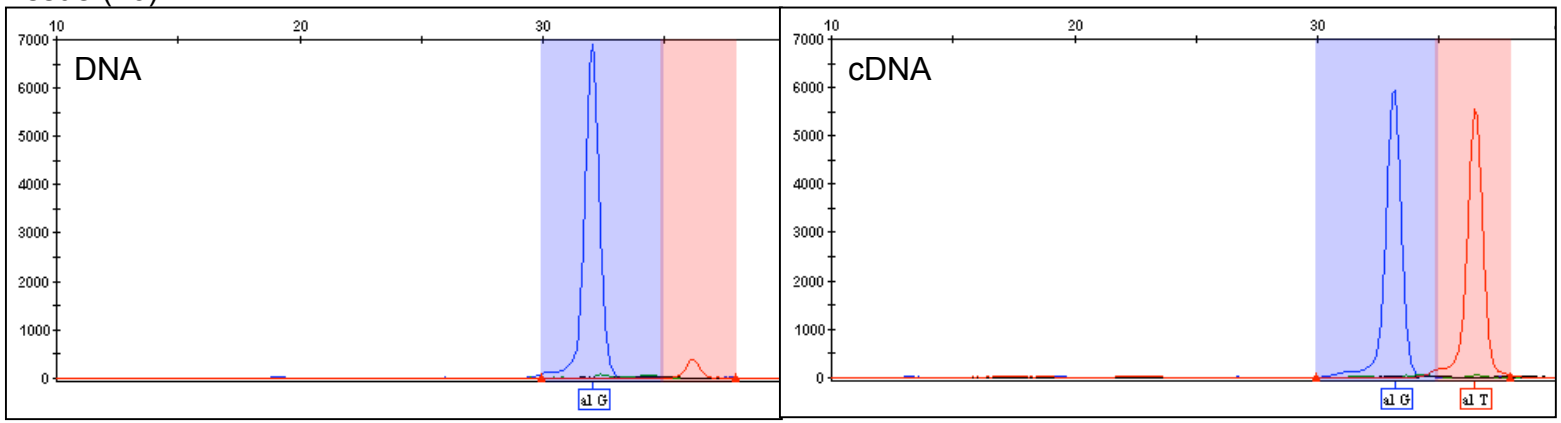
Supplementary Figure 3. SNaPshot data showing allele peaks for each TIE2 mutation

h. SNaPshot Assay, S917I Forward: g>t

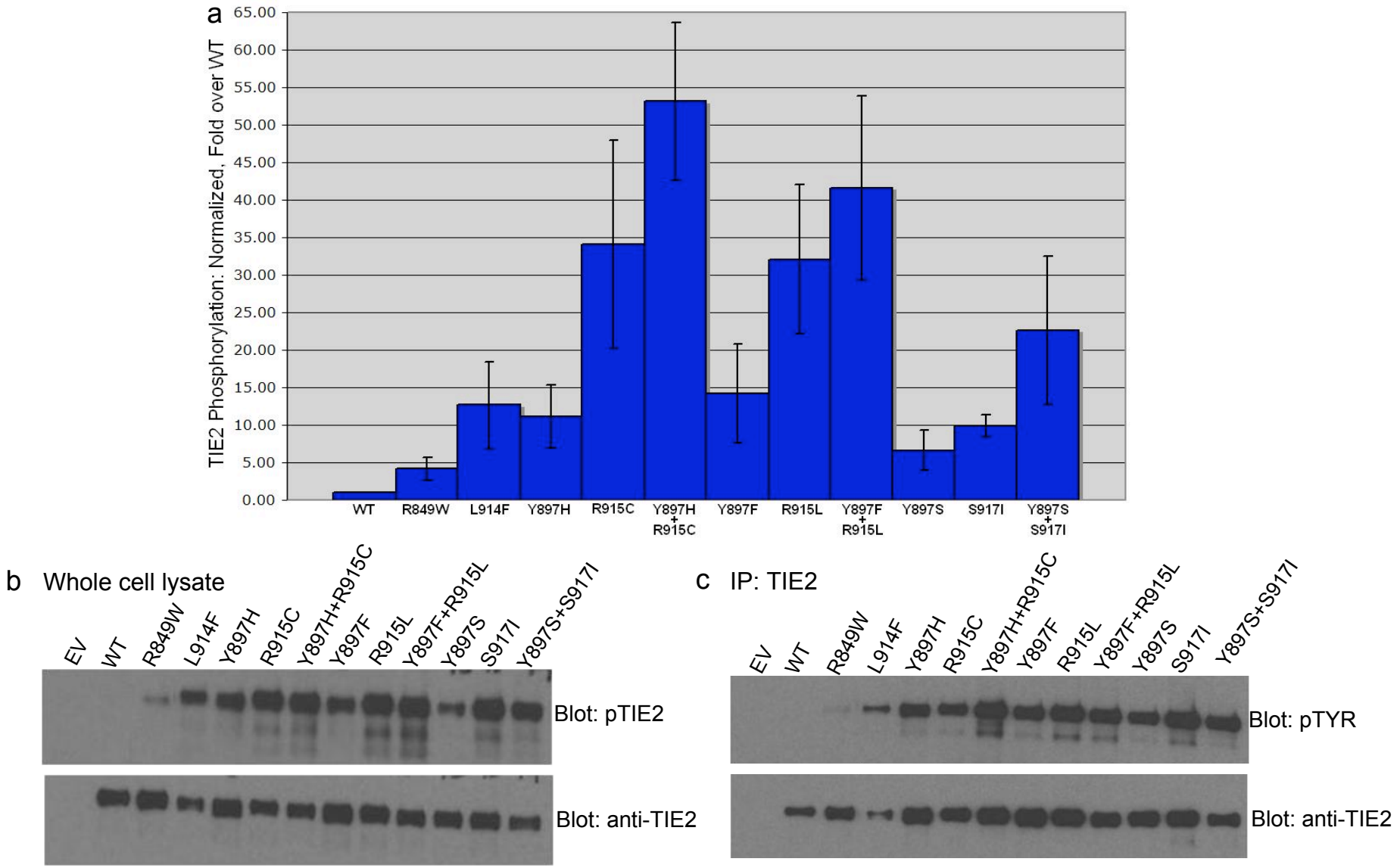
Plasmid Controls:



VM Tissue (26)

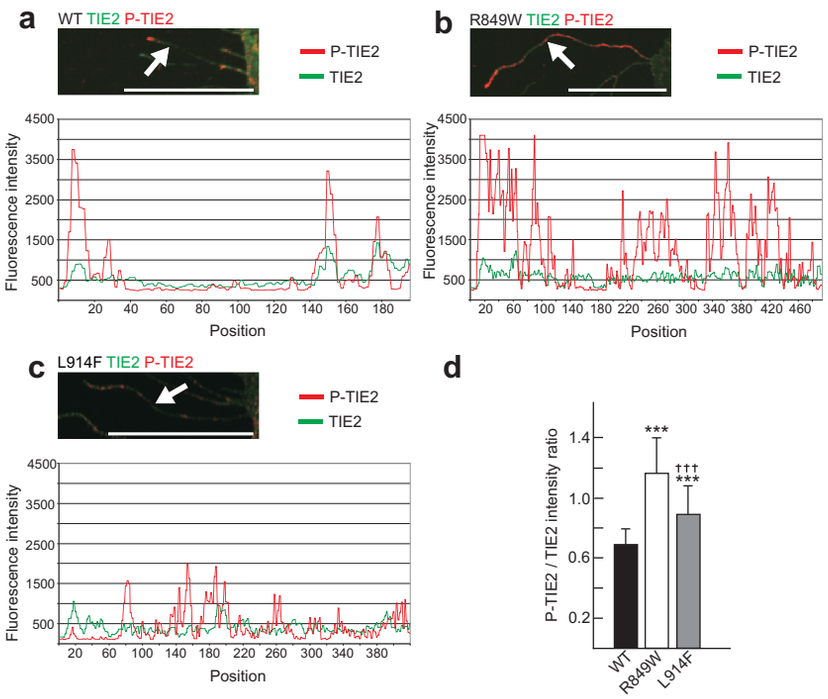


Supplementary Figure 3. (a-h) SNaPshot data showing allele peaks for each TIE2 mutant. Wild-type (WT) *vs.* mutant (Mut) plasmids, and 50:50 mixtures of these [Het] used as control templates (top panels). Data obtained from DNA and cDNA from tissue samples indicated (below). Peak colours correspond to nucleotide (allele); position of the peaks on x-axis (mobility) determined by size of single base extension-primers in combination with ddNTP added in extension reaction; relative peak-heights correlate with relative abundance of alleles detected in template (see corresponding raw peak heights obtained (**Supplementary Table 2 a-c**), and calculated relative level of mutant to wild-type alleles (**Supplementary Table 1**).



Supplementary Figure 4. TIE2 somatic mutants are hyperphosphorylated. **(a)** Graph of normalized phosphotyrosine signals (to total TIE2 when stripped and reprobed) obtained in Western blot on lysates from Cos-7 cells transfected with TIE2 mutants identified in sporadic VM. Intensities expressed as fold over wild-type (WT, set at 1) run on the same gel, across at least 3 experiments each. Common inherited VMCM mutation R849W, most frequent sporadic-mutant L914F, and three double-mutant versions of TIE2, along with constituent single mutants, assessed for relative effects on ligand-independent phosphorylation. **(b)** Confirmation of the identity of phosphorylated bands at 140kD as TIE2, by Western blots on total cell lysates from Cos-7 transfectants with anti-phosphoTIE2 (top panel) followed by strip-and-reprobe with anti-TIE2; and **(c)** Immunoprecipitation of lysates from Cos-7 transfectants with anti-TIE2, followed by Western blot with anti-phosphotyrosine, stripped and reprobed for TIE2.

Supplementary Figure 5. Fluorescence intensity measurements in retraction fibers



Supplementary Figure 5. (a-d) Fluorescence intensity measurements in retraction fibers from WT, R849W, and L914F-expressing HUVECs. TIE2 (green) and P-TIE2 (red) intensity profiles were measured along the retraction fibers (a-c, arrows). (a) In WT cells, TIE2 is typically present and phosphorylated in the distal end (position 0-20) and in nodules (position 140-160). (b) In contrast, in R849W cells, a higher P-TIE2 intensity was observed all along the fibers with areas of particularly high P-TIE2/TIE2 ratios. (c) L914F also shows activation relative to WT along retraction fibers, although lower than that observed with R849W, and without pronounced peaks of P-TIE2/TIE2 ratios. (d) Relative P-TIE2/TIE2 signal intensities in the retraction fibers are graphed as mean±S.D. Representative data from 20 cells typically containing 20-40 retraction fibers. Statistical significance was determined by Student's t-test analysis (*** $p<0.001$ vs. WT; +++ $p<0.001$ vs. R849W). Scale bar 20 µm.

Patient	Localization	Size	Lesions				Sclerosed	Mutation	Mut as % of Total		Fold enrichment
			cutaneous	sub-cutaneous	muscular	mucosal			DNA	cDNA	(cDNA/DNA)
1	para-umbilical	extensive	+	+	+			**L914F**	27.54	52.62	1.91
2	genital	extensive	+	+				**L914F**	-	-	-
3	head	extensive	+	+			+	**L914F**	4.66	66.68	14.31
4	cheek	extensive	+	+	+	+	+	**L914F**	35.82	92.23	2.57
5	lip	localized	+	+	+	+		**L914F**	9.16	18.01	1.97
6	lip	extensive	+	+	+	+	+	**L914F**	ND	48.81	ND to 48.81%
7	knee	extensive	+	+	+			**L914F**	13.73	59.67	4.35
8	knee	extensive	+	+	+			**L914F**	-	-	-
9	arm	extensive		+	+		+	**L914F**	-	-	-
10	genital-knee	extensive		+	+		+	**L914F**	48.32	88.20	1.83
11	knee	extensive	+	+	+		+	**L914F**	-	-	-
12	cubital nerve	localized	?	?	+			**L914F**	-	-	-
13	arm	extensive		+				**L914F**	-	-	-
14	thorax	localized		+	+		+	**L914F**	-	-	-
15	lip	localized				+	+	**L914F**	-	-	-
16	subclavian	localized		+				**L914F**	-	-	-
17	forehead	localized	+	+				**L914F**	-	-	-
18	lip	localized	+	+	+		+	**L914F**	-	-	-
19	cheek	localized		+			+	**L914F**	-	-	-
20	cheek	extensive		+		+	+	**L914F**	5.72	60.95	10.66
21	knee	extensive	+	+	+			**L914F**	19.66	70.59	3.59
22	knee	extensive	+	+	+			**L914F**	18.47	57.98	3.14
23	ear	localized	+	+				**L914F**	-	-	-
24	forehead	extensive	+					**L914F**	-	52.64	N/A
25	buttock	localized	+	+				**Y897H + R915C**	9.86 & 8.24	53.75 & 42.50	5.45 & 5.16
26	ear	localized	+					**Y897S + S917I**	8.21 & 6.64	61.87 & 54.30	7.54 & 8.18
27 (a)	buttock	extensive, multiple	+	+	+	+		**Y897F + R915L**	22.29 & 34.90	-	N/A
(b)	foot							**Y897F + R915L**	ND & ND	7.59 & 18.54	ND to 7.59, 18.54%
28 (a)	wrist	localized, multiple	+	+		+		**Y897C + R915C**	-	-	-
(b)	tongue							**Y897C + R915C**	4.55 & 10.79	48.69 & 44.72	10.7 & 4.14

Supplementary Table 1: VM patients with *TIE2* mutations.

(a), (b): Multiple lesions from same patient.
Mutation-detection by dHPLC-screening followed by sequencing, confirmation by allele-specific PCR or restriction-site modification.
Estimates of mutant-allele(s) as % of total (DNA or cDNA) obtained using SNaPshot assay on a subset of tissue DNA and cDNA.
"-": SNaPshot assay not performed; ND: Not detectable; N/A: Not applicable
Fold enrichment of mutant allele in cDNA as compared to DNA= (Mut as % of total cDNA / Mut as % of total DNA)

Supplementary Table 2a.

L914F

L914F- Forward: CATGGAAACCTTCTGGACTTC-C/T

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
1-Ca	L914F-CT	C	T	4150	4395	1.059	1.29	1.11	52.62	31.42	34.14
1-Cb	L914F-CT	C	T	164	125	0.762	0.93			31.46	34.15
1-Da	L914F-CT	C	T	272	84	0.309	0.38	0.38	27.54	31.58	34.35
3-Ca	L914F-CT	C	T	3296	5022	1.52	1.86	2.00	66.68	32.82	34.81
3-Cb	L914F-CT	C	T	2664	4685	1.76	2.14			32.44	34.91
3-Da	L914F-CT	C	T	2590	96	0.04	0.05	0.05	4.66	32.24	35
3-Db	L914F-CT	C	T	2734	118	0.04	0.05			32.11	35
4-Ca	L914F-CT	C	T	428	2170	5.07	6.18	11.88	92.23	32.69	35.49
4-Cb	L914F-CT	C	T	357	5144	14.41	17.57			32.07	34.91
4-Da	L914F-CT	C	T	2993	835	0.28	0.34	0.56	35.82	32.3	34.91
4-Db	L914F-CT	C	T	1985	1263	0.64	0.78			31.93	35
5-Ca	L914F-CT	C	T	3733	690	0.185	0.23	0.22	18.01	31.36	34.22
5-Cb	L914F-CT	C	T	245	43	0.176	0.21			31.37	34.15
5-Da	L914F-CT	C	T	310	28	0.090	0.11	0.10	9.16	31.5	34.26
5-Db	L914F-CT	C	T	506	38	0.075	0.09			31.58	34.25
6-Ca	L914F-CT	C	T	2859	2604	0.911	1.11	0.95	48.81	31.42	34.24
6-Cb	L914F-CT	C	T	167	109	0.653	0.80			31.37	34.15
6-Da	L914F-CT	C	-	413	-	0.000	0.00	0.00	0.00	31.5	-
6-Db	L914F-CT	C	-	573	-	0.000	0.00			31.56	-
7-Ca	L914F-CT	C	T	3114	4335	1.392	1.70	1.48	59.67	31.46	34.22
7-Cb	L914F-CT	C	T	145	150	1.034	1.26			31.42	34.14
7-Da	L914F-CT	C	T	407	49	0.120	0.15	0.16	13.73	31.45	34.26
7-Db	L914F-CT	C	T	427	60	0.141	0.17			31.44	34.17
10-Ca	L914F-CT	C	T	713	3402	4.77	5.82	7.47	88.20	32.59	35.49
10-Cb	L914F-CT	C	T	228	1706	7.48	9.12			32.75	34.73
10-Da	L914F-CT	C	T	1358	1070	0.79	0.96	0.94	48.32	32.19	35
10-Db	L914F-CT	C	T	2630	1961	0.75	0.91			32.41	35
20-Ca	L914F-CT	C	T	3496	4914	1.406	1.71	1.56	60.95	31.54	34.34
20-Cb	L914F-CT	C	T	123	142	1.154	1.41			31.46	34.23
20-Da	L914F-CT	C	T	314	14	0.045	0.05	0.06	5.72	31.56	34.25
20-Db	L914F-CT	C	T	456	25	0.055	0.07			31.61	34.45
21-Ca	L914F-CT	C	T	2233	4035	1.81	2.20	2.40	70.59	32.7	34.81
21-Cb	L914F-CT	C	T	2031	4325	2.13	2.60			32.98	34.73
21-Da	L914F-CT	C	T	2396	547	0.23	0.28	0.24	19.66	32.15	35
21-Db	L914F-CT	C	T	2826	489	0.17	0.21			32.44	35
22-Ca	L914F-CT	C	T	3307	4307	1.302	1.59	1.38	57.98	31.46	34.22
22-Cb	L914F-CT	C	T	151	145	0.960	1.17			31.37	34.15
22-Da	L914F-CT	C	T	406	76	0.187	0.23	0.23	18.47	31.48	34.27
22-Db	L914F-CT	C	T	472	87	0.184	0.22			31.68	34.44
24-Ca	L914F-CT	C	T	782	709	0.91	1.11	1.11	52.64	31.31	34.35
24-Cb	L914F-CT	C	T	1229	1126	0.92	1.12			31.58	34.44
Het-1	L914F-CT	C	T	237	225	0.95	Avg when 50:50=			31.86	34.7
Het-2	L914F-CT	C	T	578	402	0.70	0.82			31.43	34.43
Mut-1	L914F-CT	-	T	-	819						34.8
Mut-2	L914F-CT	-	T	-	780						34.26
WT-1	L914F-CT	C	-	459	-					31.98	
WT-2	L914F-CT	C	-	1435	-					31.15	

Supplementary Table 2: SNaPshot data showing peak sizes (positions on the x-axis: mobility) and heights of wild-type (WT; black) and mutant (Mut; red) alleles.

Ca: cDNA amplicon 1; Cb: cDNA amplicon 2; Da: DNA amplicon 1, Db: DNA amplicon 2

Yellow highlight: Plasmid-DNA Ctrls for: wild-type (WT), Mutant (Mut), and 50:50 ratio of wild-type:mutant alleles (Het-1 and 2)

Mut/WT Allele Ratio= Peak height (Mut)/ Peak height (WT); Normalized Ratio= [(Mut/WT allele Ratio)/(Avg when 50:50)]; where Avg when 50:50= [Avg (Mut/WT Allele Ratio) from Het-1 & Het-2]

Mutant (% of Total) = [Normalized Mut/Wt ratio X (100/{Normalized mut/wt ratio+1})]

Supplementary Table 2b.

Y897H

Y897H-Reverse: GTACTCAATGGCCAGGTACAAGT-A/G

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
25-Ca	Y897H-AG	A	G	1555	3583	2.304	1.213	1.162	53.75	36.37	33.99
25-Cb	Y897H-AG	A	G	1726	3646	2.112	1.112			35.49	33.61
25-Da	Y897H-AG	A	G	4353	883	0.203	0.107	0.109	9.86	36.38	34.1
25-Db	Y897H-AG	A	G	4399	937	0.213	0.112			36.27	34.03
Het-1	Y897H-AG	A	G	2644	5918	2.24	Avg when 50:50=			35.59	33.77
Het-2	Y897H-AG	A	G	1280	1990	1.55	1.90			35.58	33.68
Mut	Y897H-AG	-	G	-	4434					-	33.69
WT	Y897H-AG	A	-	2456	-					35.59	-

Y897S

Y897C/F/S-Reverse: CGTACTCAATGGCCAGGTACAAG-T/G

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
26-Ca	Y897S-TG	T	G	1342	5390	4.016	1.575	1.622	61.87	35.81	32.97
26-Cb	Y897S-TG	T	G	843	3589	4.257	1.670			35.71	32.92
26-Da	Y897S-TG	T	G	5608	1230	0.219	0.086	0.089	8.21	35.71	32.99
26-Db	Y897S-TG	T	G	6294	1489	0.237	0.093			35.71	32.89
Het-1	Y897S-TG	T	G	2046	5236	2.56	Avg when 50:50=			35.69	33.48
Het-2	Y897S-TG	T	G	862	2197	2.55	2.55			35.78	33.49
Mut	Y897S-TG	-	G	-	6705					-	33.53
WT	Y897S-TG	T	-	1592	-					35.78	-

Y897F

Y897C/F/S-Reverse: CGTACTCAATGGCCAGGTACAAG-T/A

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
27(a)-Db	Y897F-TA	T	A	4396	2033	0.462	0.300	0.287	22.29	36.56	36.56
27(a)-Da	Y897F-TA	T	A	7027	2958	0.421	0.273			36	35.9
27(b)-Ca	Y897F-TA	T	A	6775	871	0.129	0.083	0.082	7.59	35.8	35.6
27(b)-Cb	Y897F-TA	T	A	5217	649	0.124	0.081			35.8	35.6
27(b)-Da	Y897F-TA	T	-	6722	-	0.000	0.000	0.000	0.00	36.2	-
27(b)-Db	Y897F-TA	T	-	4772	-	0.000	0.000			36.57	-
Het-1	Y897F-TA	T	A	763	1147	1.50	Avg when 50:50=			36	35.8
Het-2	Y897F-TA	T	A	997	1575	1.58	1.54			36.14	35.93
Mut-1	Y897F-TA	-	A	-	2513					-	35.9
Mut-2	Y897F-TA	-	A	-	2389					-	35.82
WT-1	Y897F-TA	T	-	826	-					35.91	-
WT-2	Y897F-TA	T	-	1081	-					36.09	-

Y897C

Y897C/F/S-Reverse: CGTACTCAATGGCCAGGTACAAG-T/C

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
28(b)-Ca	Y897C-TC	T	C	6797	4284	0.630	0.810	0.949	48.69	35.79	34.33
28(b)-Cb	Y897C-TC	T	C	1488	1259	0.846	1.088			35.78	34.33
28(b)-Da	Y897C-TC	T	C	6995	271	0.039	0.050	0.048	4.55	35.92	34.04
28(b)-Db	Y897C-TC	T	C	7677	272	0.035	0.046			35.71	33.87
Het-1	Y897C-TC	T	C	425	330	0.776	Avg when 50:50=			35.5	33.5
Het-2	Y897C-TC	T	C	440	343	0.780	0.778			35.5	33.53
Mut	Y897C-TC	-	C	-	554					-	33.28
WT	Y897C-TC	T	-	1324	-					35.5	-

Supplementary Table 2: SNaPshot data showing peak sizes (positions on the x-axis: mobility) and heights of wild-type (WT; black) and mutant (Mut; red) alleles.

Ca: cDNA amplicon 1; Cb: cDNA amplicon 2; Da: DNA amplicon 1, Db: DNA amplicon 2

Yellow highlight: Plasmid-DNA Ctrls for: wild-type (WT), Mutant (Mut), and 50:50 ratio of wild-type:mutant alleles (Het-1 and 2)

Mut/WT Allele Ratio= Peak height (Mut)/ Peak height (WT); Normalized Ratio= [(Mut/WT allele Ratio)/(Avg when 50:50)]; where Avg when 50:50= [Avg (Mut/WT Allele Ratio) from Het-1 & Het-2]

Mutant (% of Total) = [Normalized Mut/Wt ratio X (100/{Normalized mut/wt ratio+1})]

Supplementary Table 2c.

R915C

R915C-Reverse: TCCGTCTCCAGCACACGGCTCTTGC-G/A

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
25-Ca	R915C-GA	G	A	4382	3145	0.718	0.704	0.739	42.50	32.67	34.23
25-Cb	R915C-GA	G	A	4030	3184	0.790	0.775			33.29	34.62
25-Da	R915C-GA	G	A	6874	676	0.098	0.096	0.090	8.24	32.46	34.24
25-Db	R915C-GA	G	A	5866	498	0.085	0.083			32.39	34.08
28(b)-Ca	R915C-GA	G	A	5599	4092	0.731	0.717	0.809	44.72	32.56	34.22
28(b)-Cb	R915C-GA	G	A	4588	4217	0.919	0.901			33.27	34.62
28(b)-Db	R915C-GA	G	A	5757	708	0.123	0.121	0.121	10.79	32.17	34.19
Het-1	R915C-GA	G	A	4474	4831	1.08	Avg when 50:50=			33.32	34.63
Het-2	R915C-GA	G	A	1920	1854	0.97	1.02			33.22	34.63
Mut	R915C-GA	-	A	-	3243					-	34.63
WT	R915C-GA	G	-	4069	-					33.16	-

R915L

R915L-Reverse: GTCTCCAGCACACGGCTCTTG-C/A

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
27(a)-Da	R915L-CA	C	A	2255	1923	0.853	0.572	0.536	34.90	32.34	35.6
27(a)-Db	R915L-CA	C	A	5073	3777	0.745	0.500			32.93	35.7
27(b)-Ca	R915L-CA	C	A	2885	808	0.280	0.188	0.228	18.54	32.5	35.7
27(b)-Cb	R915L-CA	C	A	2549	1015	0.398	0.267			32.46	35.71
27(b)-Da	R915L-CA	C	-	2478	-	0.000	0.000	0.000	0.00	32.63	-
27(b)-Db	R915L-CA	C	-	4637	-	0.000	0.000			32.8	-
Het-1	R915L-CA	C	A	680	988	1.45	Avg when 50:50=			32.17	35.83
Het-2	R915L-CA	C	A	1707	2593	1.52	1.49			31.54	35.31
Mut	R915L-CA	-	A	-	2410					-	35.73
WT	R915L-CA	C	-	1140	-					32.02	-

S917I

S917I-Forward: AACCTTCTGGACTTCCTTCGCAAGA-G/T

Sample Name	Marker	Allele 1 (WT)	Allele 2 (Mut)	Peak Height (WT)	Peak Height (Mut)	Mut/WT Allele Ratio	Normalized Ratio	Avg	Mut (% of Total)	Peak Size (WT)	Peak Size (Mut)
26-Ca	S917I-GT	G	T	5951	5563	0.935	1.154	1.188	54.30	33.17	36.45
26-Cb	S917I-GT	G	T	4063	4023	0.990	1.222			33.2	36.56
26-Da	S917I-GT	G	T	4514	265	0.059	0.072	0.071	6.64	32.31	36.2
26-Db	S917I-GT	G	T	6911	390	0.056	0.070			32.01	36.11
Het-1	S917I-GT	G	T	1933	1692	0.88	Avg when 50:50=			33.24	36.49
Het-2	S917I-GT	G	T	1842	1378	0.75	0.81			33.11	36.48
Mut	S917I-GT	-	T	-	2687					-	36.46
WT	S917I-GT	G	-	4171	-					33.18	-

Supplementary Table 2: SNaPshot data showing peak sizes (positions on the x-axis: mobility) and heights of wild-type (WT; black) and mutant (Mut; red) alleles.

Ca: cDNA amplicon 1; Cb: cDNA amplicon 2; Da: DNA amplicon 1, Db: DNA amplicon 2

Yellow highlight: Plasmid-DNA Ctrls for: wild-type (WT), Mutant (Mut), and 50:50 ratio of wild-type:mutant alleles (Het-1 and 2)

Mut/WT Allele Ratio= Peak height (Mut)/ Peak height (WT); Normalized Ratio= [(Mut/WT allele Ratio)/(Avg when 50:50)]; where Avg when 50:50= [Avg (Mut/WT Allele Ratio) from Het-1 & Het-2]

Mutant (% of Total) = [Normalized Mut/Wt ratio X (100/{Normalized mut/wt ratio+1})]

Supplementary Table 3a: Bioinformatic analyses of TIE2 mutations

Nt change	AA change	Predicted effect	dbSNP	dbEST	dbESE	POLYPHEN	PANTHER (subSPEC value)
c.2689 t>c	p.Y897H	Missense	Absent	Absent	No change	Possibly damaging	High probablity of deleterious effect (-3.96)
c.2690 a>c	p.Y897S	Missense	Absent	Absent	No change	Probably damaging	High probablity of deleterious effect (-3.77)
c.2690 a>t	p.Y897F	Missense	Absent	Absent	No change	Benign	Unlikely deleterious effect (-2.11)
c.2690 a>g	p.Y897C	Missense	Absent	Absent	No change	Probably damaging	High probability of deleterious effect (-4.96)
c.2740 c>t	p.L914F	Missense	Absent	Absent	No change	Benign	Possible deleterious effect (-3,21)
c.2743 c>t	p.R915C	Missense	Absent	Absent	No change	Probably damaging	High probability of deleterious effect (-5,3)
c.2744 g>t	p.R915L	Missense	Absent	Absent	deletion of one ESE site	Probably damaging	High probablity of deleterious effect (-3,91)
c.2750 g>t	p.S917I	Missense	Absent	Absent	(+)2	Benign	High probablity of deleterious effect (-3,79)

subSPEC value : substitution Position-Specific Evolutionary Conservation (<-3.5=high probability of deleterious functional effect; -2.5 to -3.5=possible deleterious functional effect;-2.5 to 2.5=unlikely functional effect; 2.5-3.5=possible gain-of-function effect; >3.5=high probability of gain-of-function effect)

Supplementary Table 3b: Conservation of amino acid residues Y897, L914, R915 and S917 across tyrosine kinase receptors in different species

```
                                  *                 ***
                                  897               914 915 917
TIE2_Human          LGACEH-RGYLYLAIEYAPHGNLLDFLRKSRVL
TIE2_Mouse          LGACEH-RGYLYLAIEYAPHGNLLDFLRKSRVL
TIE2_Bovin          LGACEH-RGYLYLAIEYAPHGNLLDFLRKSRVL
TIE2_Brare          LGACEH-RGYLYLAIEFAPHGNLLDFLRKSRVL
TIE1_Human          LGACKN-RGYLYIAIEYAPYGNLLDFLRKSRVL
TIE1_Mouse          LGACEN-RGYLYIAIEYAPYGNLLDFLRKSRVL
TIE1_Bovin          LGACEN-RGYLYIAIEYAPYGNLLDFLRKSRVL
FGFR1_Human         LGACTQ-DGPLYVIVEYASKGNLREYLQARRP-
FGFR2_Human         LGACTQ-DGPLYVIVEYASKGNLREYLRARRP-
FGFR3_Human         LGACTQ-GGPLYVLVEYAAKGNLREFLRARRP-
VEGFR1_Human        LGACTKQGGPLMVIVEYCKYGNLSNYLKSKRDL
VEGFR2_Human        LGACTKPGGPLMVIVEFCKFGNLSTYLRSKRNE
VEGFR3_Human        LGACTKPQGPLMVIVEFCKYGNLSNFLRAKRDA
```

Supplementary Methods

Computational analysis. Each nucleotide change identified in the coding regions of *TIE2* was subjected to a series of bioinformatic analyses. First, dbSNP and dbEST were searched to detect known polymorphisms. Changes modifying an amino acid codon were submitted to PANTHER (http://www.pantherdb.org) and POLYPHEN (http://www.bork.embl-heidelberg.de/PolyPhen/) to estimate the likelihood of functional impact on the protein based on the position-specific evolutionary conservation in related proteins. RESCUE-ESE (Exonic Splicing Enhancer) was used to predict splicing alterations (http://genes.mit.edu/burgelab/rescue-ese).

URLs. The following URLs for the databases and softwares were used:
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/; University Of California-Santa Cruz (UCSC) Human Genome Bioinformatics, http://genome.ucsc.edu/; dbSNP, http://www.ncbi.nlm.nih.gov/SNP/; dbEST, http://www.ncbi.nih.gov/EST/; Exonic Splicing Enhancer (ESE), http://genes.mit.edu/burgelab/rescue-ese/; ClustalW, http://www.ebi.ac.uk/clustalw/, Ensembl, http://www.ensembl.org; PANTHER, http://www.pantherdb.org; POLYPHEN, http://www.bork.embl-heidelberg.de/PolyPhen; ESE databases (Exonic Splicing Enhancer, http://genes.mit.edu/burgelab/rescue-ese/.

Cos-7 Cell Culture and transfection. Cos-7 cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and Penicillin G 100 U/ml /Streptomycin 100 μg/ml (Cambrex Bioscience). They were transfected with full-length wild-type or mutant(s) TIE2

expression constructs using jetPEI (Polyplus-transfections SA), according to manufacturer's instructions. Co-transfections were done similarly, with different ratios of R849W and the deletion-mutant as indicated.

Cell lysis. Cells were washed with ice-cold PBS, and lysed in cold lysis buffer [(20 mM Hepes pH7.8, 75 mM KCl, 0.1 mM EDTA, 1 mM sodium-orthovanadate *or* 2 tablets of PhosStop phosphatase inhibitor tablets (Roche), 2 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5% Triton-X 100 and 1 tablet of Complete Protease Inhibitor (Roche) per 20 ml]. Lysates were subjected to sonication and spun at 13000X g to remove debris.

Western blot. Supernatants were resolved on a polyacrylamide gel and immunoblotted with an anti-phospho-tyrosine antibody (PY99; Santa Cruz) at 1:400 [or anti-phospho-Tie2$^{1102/1108}$ (Ab-1, Calbiochem) at 1:2,500, in confirmatory blots], and stripped and re-probed with anti-TIE2 antibody (C-20; Santa Cruz Biotechnologies) at 1:500 dilution. Immunoreactive proteins were visualized using a goat anti-rabbit-HRP secondary antibody (for C-20 and Ab-1) at 1:25000 (Biosource) or a goat anti-mouse-HRP antibody (for PY99) at 1:20,000 (Biosource), with a femto-range-sensitive ECL detection system (Pierce).

Densitometric image-quantification. Densitometric analysis was carried out using ImageJ (http://rsb.info.nih.gov/ij/). Phosphotyrosine signals of bands were normalized to the corresponding total-TIE2 signals when stripped and reprobed, and the fold-differences of normalized (mutant) phosphorylation over that of wild-type TIE2 (run on the same gel) were calculated. Average fold-differences over WT observed across at least 3 separate blots per mutant form, were graphed.

Ang1 binding and immunoprecipitation. Transiently transfected Cos-7 cells in 6-well plates were incubated in serum-low (0.5% FCS) media for 3 hours, at 24 hours post-transfection. His-tagged Ang1 (R&D Systems) was added to wells at 1microg/mL in the media, and plates were incubated on ice at 4° for 30 minutes. Cells were lysed using 0.05M Tris-HCl, 0.15M NaCl, 1% NP40, 2mM EDTA, with 1 Complete tablet and 2 PhosStop tablets. Lysates were homogenized using 21G needles, and spun to remove debris. They were pre-cleared with 25 microL 50% Sepharose A beads (Sigma-Aldrich), and immunoprecipitated using 10microg/ml anti-His antibody with 25 microl Sepharose A beads. Direct immunoprecipitation of TIE2 was instead performed using anti-TIE2 (C-20, Santa Cruz; 2.5microg/ml). Western blot was performed as described, for anti-phosphotyrosine (PY99), followed by strip and reprobe for anti-TIE2.

Quantification of fluorescence intensity in HUVEC retraction fibers. To measure fluorescence intensity within retraction fibers, they were chosen as regions of interest (ROIs) using digitalized unsaturated confocal microscope images. For quantification, the fluorescence intensities of Cy2 and Cy3 immunofluorescences in ROIs were measured by Fluoview software.