Leong et al., http://www.jem.org/cgi/content/full/jem.20071082/DC1

SUPPLEMENTAL MATERIALS AND METHODS

Immunoblotting. Concentrated supernatant from cultured cells was obtained by filtering 3-d-conditioned medium through a 100,000-kD cutoff ultrafiltration membrane (Millipore).

Flow cytometry. MCF-10A cell lines were detached by incubation in PBS-based enzyme-free cell dissociation buffer (Invitrogen). Cells were stained with mouse monoclonal antibody to surface E-cadherin (Chemicon), followed by goat anti–mouse Alexa Fluor 594 (Invitrogen) secondary antibody. Samples were run on a flow cytometer (EPICS ELITE-ESP; Beckman Coulter), and data were analyzed with WinList software (Verity Software House Inc.).

Methylation assays. For global genomic DNA methylation analysis, 500 ng of genomic DNA was digested with the restriction enzymes HpaII, MspI, or McrBC (all obtained from New England Biolabs, Inc.). HpaII is unable to digest CpG-methylated DNA, whereas its isoschizomer MspI is not sensitive to CpG methylation and, thus, is a positive control for DNA digestion. McrBC digests CpG-methylated DNA only in the presence of GTP. For McrBC digestion, reactions were performed with GTP (McrBC-plus-GTP) or without GTP (McrBC-minus-GTP). Entire reaction mixtures were assessed in 2% TAE-agarose gels containing ethidium bromide. For HpaII and McrBC-plus-GTP reactions, digested DNA products above 6 kb were quantitated by densitometry. For control and McrBC-minus-GTP reactions, undigested DNA products corresponding to the topmost DNA band were quantitated by densitometry. Data are expressed as a ratio of digested over undigested DNA products (digested/undigested ratio) and represent the mean ratio + SEM from five control tumors and five XNotch4 tumors.

Immunostaining. 7-μm-thick tumor cryosections were stained with hematoxylin and eosin, rabbit polyclonal antibody to HA (BAbCo), and rat monoclonal antibody to mouse CD31 (BD Biosciences). For quantitation of the percentage of CD31-stained area, at least six random fields at 200× magnification were analyzed per tumor using Northern Eclipse software. Vascular density was quantitated by expressing the CD31-stained area as a percentage of the total tumor area. For quantitation of the number of vessels per square millimeter, entire tumor sections were analyzed using Northern Eclipse software. Data are expressed as the mean percentage of CD31-stained area + SEM from 14 control tumors and 12 XNotch4 tumors, and the mean number of vessels per square millimeter + SEM from 13 control tumors and 16 XNotch4 tumors.

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Table S1. PCR primer sequences

Gene	Primer identifier[a]	Species specificity		Primer sequences (5′ to 3′)		Reference
		Human	Mouse			
Jagged1	A	X		Forward:	CTATGATGAGGGGGATGCT	1
	B		X	Forward:	AATGGAGACTCCTTCACCTGT	2
	C	X	X	Reverse:	CGTCCATTCAGGCACTGG	2
Jagged2	A	X		Forward:	TGGGATGCCTGGCACA	This study
	B		X	Forward:	CAGGGCACGCGGTGT	This study
	C	X	X	Reverse:	CCGGCAGATGCAGGA	This study
Delta-like1	A	X		Forward:	GAGGGAGGCCTCGTGGA	This study
	B		X	Forward:	TGGTTCTCTCAGAGTTAGCAGAG	This study
	C	X	X	Reverse:	AGACCCGAAGTGCCTTTGTA	This study
Delta-like3	A	X	X	Forward:	CGGATGCACTCAACAACCT	This study
	B	X		Reverse:	GAAGATGGCAGGTAGCTCAA	This study
	C		X	Reverse:	ATAGATGTCTCTGGGGAGATGA	This study
Delta-like4	A	X	X	Forward:	GCATTGTTTACATTGCATCCTG	This study
	B	X		Reverse:	GCAAACCCCAGCAAGAGAC	This study
	C		X	Reverse:	GTAGCTCCTGCTTAATGCCAAA	This study
Notch1	A	X		Forward:	CACTGTGGGCGGGTCC	3
	B	X		Reverse:	GTTGTATTGGTTCGGCACCAT	3
	C		X	Forward:	GGCCACCTCTTCACTGCTTC	2
	D	X	X	Reverse:	CCGGAACTTCTTGGTCTCCA	2
Notch2	A	X		Forward:	AATCCCTGACTCCAGAACG	This study
	B		X	Forward:	AACTGGAGAGTCCAAGAAACG	2
	C	X	X	Reverse:	TGGTAGACCAAGTCTGTGATGAT	2
Notch3	A	X		Forward:	TGAGACGCTCGTCAGTTCTT	1
	B		X	Forward:	CACCTTGGCCCCCTAAG	2
	C	X	X	Reverse:	TGGAATGCAGTGAAGTGAGG	2
Notch4	A	X		Forward:	TAGGGCTCCCCAGCTCTC	1
	B		X	Forward:	CAAGCTCCCGTAGTCCTACTTC	2
	C	X	X	Reverse:	GGCAGGTGCCCCCATT	2
HES1	A	X		Forward:	AGGCGGACATTCTGGAAATG	4
	B	X		Reverse:	CGGTACTTCCCCAGCACACTT	4
HEY1	A	X		Forward:	GGAGAGGCGCCGCTGTAGTTA	5
	B	X		Reverse:	CAAGGGCGTGCGCGTCAAAGTA	5
	C	X	X	Forward:	GAGAAGCAGGGATCTGCTAA	This study
	D	X	X	Reverse:	CCCAAACTCCGATAGTCCAT	This study
HEY2	A	X	X	Forward:	ACAGGGGGTAAAGGCTACTTTG	This study
	B	X		Reverse:	CTGCTGCTGCTGCGTTT	This study
	C	X	X	Reverse:	GAAGGACAGAGGGAAGCTGTGTG	5
HEYL	A	X	X	Forward:	TCCCCACTGCCTTTGAG	This study
	B	X		Reverse:	CTGCTGGGGGCGACA	This study
	C	X	X	Reverse:	GGCACTCTTCCCAGGAT	This study
E-cadherin	A	X		Forward:	CAGCACGTACACAGCCCTAA	6
	B	X		Reverse:	ACCTGAGGCTTTGGATTCCT	6
	C	X		Forward:	TTAGGTTAGAGGGTTATCGCGT	7
	D	X		Reverse:	TAACTAAAAATTCACCTACCGAC	7
	E	X		Forward:	TAATTTTAGGTTAGAGGGTTATTGT	7
	F	X		Reverse:	CACAACCAATCAACAACACA	7
	G	X		Forward:	GTTTAGTTTTGGGGAGGGGTT	8
	H	X		Reverse:	ACTACTACTCCAAAAACCCATAACTAA	8
Slug	A	X		Forward:	AGATGCATATTCGGACCCAC	6
	B	X		Reverse:	CCTCATGTTTGTGCAGGAGA	6
Snail	A	X		Forward:	AATCGGAAGCCTAACTACAGCGAG	9
	B	X		Reverse:	CCTTGGCCTCAGAGAGCTGG	9
SIP1	A	X		Forward:	ACACCCCTGGCACAACAA	This study
	B	X		Reverse:	GTGTCACTGCGCTGAAGGTA	This study
Twist1	A	X		Forward:	CACTGAAAGGAAAGGCATCA	10
	B	X		Reverse:	GGCCAGTTTGATCCCAGTAT	10
GAPDH	A	X		Forward:	GGACCTGACCTGCCGTCTAGAA	11
	B	X		Reverse:	GGTGTCGCTGTTGAAGTCAGAG	11
	C		X	Forward:	AATGTGTCCGTCGTGGATCT	12
	D		X	Reverse:	CCCTGTTGCTGTAGCCGTAT	12
	E	X	X	Forward:	CCCATCACCATCTTCCAG	13
	F	X	X	Reverse:	ATGACCTTGCCCACAGCC	13

[a]The primer identifier is a letter assigned to each individual primer. Various combinations of two primers (Table S2, Primer set) are used to amplify the target gene of interest.

Table S2. PCR primer sets and conditions

Gene	Primer set[a]	Annealing temperature (°C)	Product size (bp)
Human Jagged1	A/C	53	507
Mouse Jagged1	B/C	53	383
Human Jagged2	A/C	53	550
Mouse Jagged2	B/C	58	550
Human Delta-like1	A/C	55	448
Mouse Delta-like1	B/C	55	410
Human Delta-like3	A/B	55	338
Mouse Delta-like3	A/C	55	329
Human Delta-like4	A/B	60	456
Mouse Delta-like4	A/C	55	473
Human Notch1	A/B	55	85
Mouse Notch1	C/D	60	529
Human Notch2	A/C	53	589
Mouse Notch2	B/C	53	583
Human Notch3	A/C	53	667
Mouse Notch3	B/C	60	449
Human Notch4	A/C	60	486
Mouse Notch4	B/C	53	486
Human CSL	A/B	57	123
Human HES1	A/B	55	103
Human HEY1	A/B	57	428
Human and mouse HEY1	C/D	53	137
Human HEY2	A/B	57	574
Human and mouse HEY2	A/C	53	531
Human HEYL	A/B	53	583
Human and mouse HEYL	A/C	53	391
Human E-cadherin	A/B	53	159
Human E-cadherin-M	C/D	57	116
Human E-cadherin U	E/F	53	97
Human E-cadherin–promoter sequencing	G/H	50	270
Human Slug	A/B	53	258
Human Snail	A/B	50	400
Human SIP1	A/B	53	234
Human GAPDH	A/B	53	142
Mouse GAPDH	C/D	53	256
Human and mouse GAPDH	E/F	53	446

[a]The primer set is a primer identifier pair (see Table S1 for individual primer identifiers) used to amplify the target gene of interest.

Table S3. Gene accession numbers associated with Oncomine microarray data sets

Gene	Accession no.	Breast 1	Breast 2	Adrenal	Brain	Endocrine	Gastric	Lung	Ovarian	Renal	Salivary	Sarcoma 1	Sarcoma 2
Jagged1	NM_000214	X											
Jagged1	AA933616		X										
Jagged1	R70684		X				X	X	X	X		X	
Jagged1	U77914			X	X	X					X		X
Jagged1	AI378220			X							X		
Jagged1	U73936				X								
Jagged1	U61276				X								
Jagged1	AI457817				X								
Jagged1	T96855							X					
Slug	AI572079	X											
Slug	H57309		X				X	X	X	X		X	
Slug	N91754		X				X	X	X	X		X	
Slug	U69196			X		X					X		X
Slug	NM_003068				X								
Snail	NM_005985	X											
Snail	AA465052							X	X	X			
Notch1	NM_017617	X											
Notch1	AA903201		X										
Notch1	H18865		X										
HEY1	NM_012258	X											
HEY1	R61374		X										
HEY2	NM_012259	X											
HEY2	AI299482		X										
HEYL	AL040198	X											
HEYL	AA969508		X										
HEYL	R27319		X										

X indicates accession numbers associated with the particular data set.