Simultaneous Detection of Akt and MAPK Signaling Pathway Targets Reveals Reciprocal Regulation of Phospho-Akt(S473) and Phospho-S6RP(S235/236 and S240/244) as a Function of Cell Density

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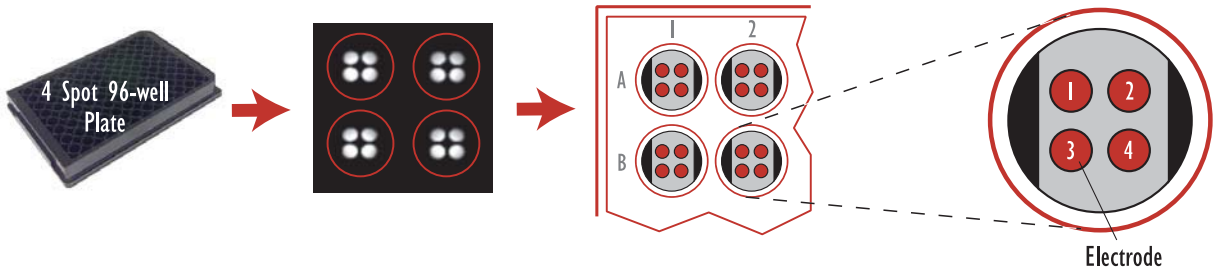
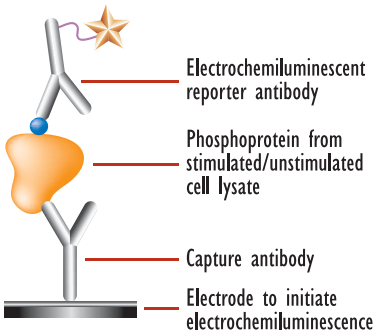
Cell density is a potent regulator of the cell cycle during exponential growth and thus affects gene expression. Many recent studies have characterized cell-density as a controlling factor for cell-cell interactions and the binding of surface-associated adhesion molecules to the cytoskeleton. We examined the impact of cell density on two distinct signal transduction pathways, Protein kinase B (Akt) and Mitogen-activated protein kinases (MAPK). These pathways regulate cell proliferation, differentiation, and apoptosis, and exhibit cross-talk. Downstream, the MAPK and Akt pathways regulate p70S6kinase (p70S6K) and its substrate S6 ribosomal protein (S6RP). Whereas levels of phospho-S6RP are known to be regulated by phosphorylated Akt, our findings suggest that in Jurkat cells this is dependent upon the cell density. We show that maximum phosphorylation of S6RP(S235/236 and S240/244) is observed at lower cell densities ($0.5x10^6$ cells/mL). In contrast, levels of phosphorylated Akt increase with higher cell densities ($1.3x10^6$ cells/mL). In order to study the impact of cell density over a wide range of targets in the two pathways, we have developed highly sensitive multiplex assays that detect multiple phosphoproteins simultaneously in a single well with low microgram levels of cell lysate protein. Low and high density Jurkat cells were interrogated for the phosphorylation status in multiplex panels of Akt/p70S6K/GSK-3β and ERK/JNK/p38; the results from these studies will be presented.

MSD® MULTI-ARRAY® Technology and MULTI-SPOT® Plates



Assay Protocol

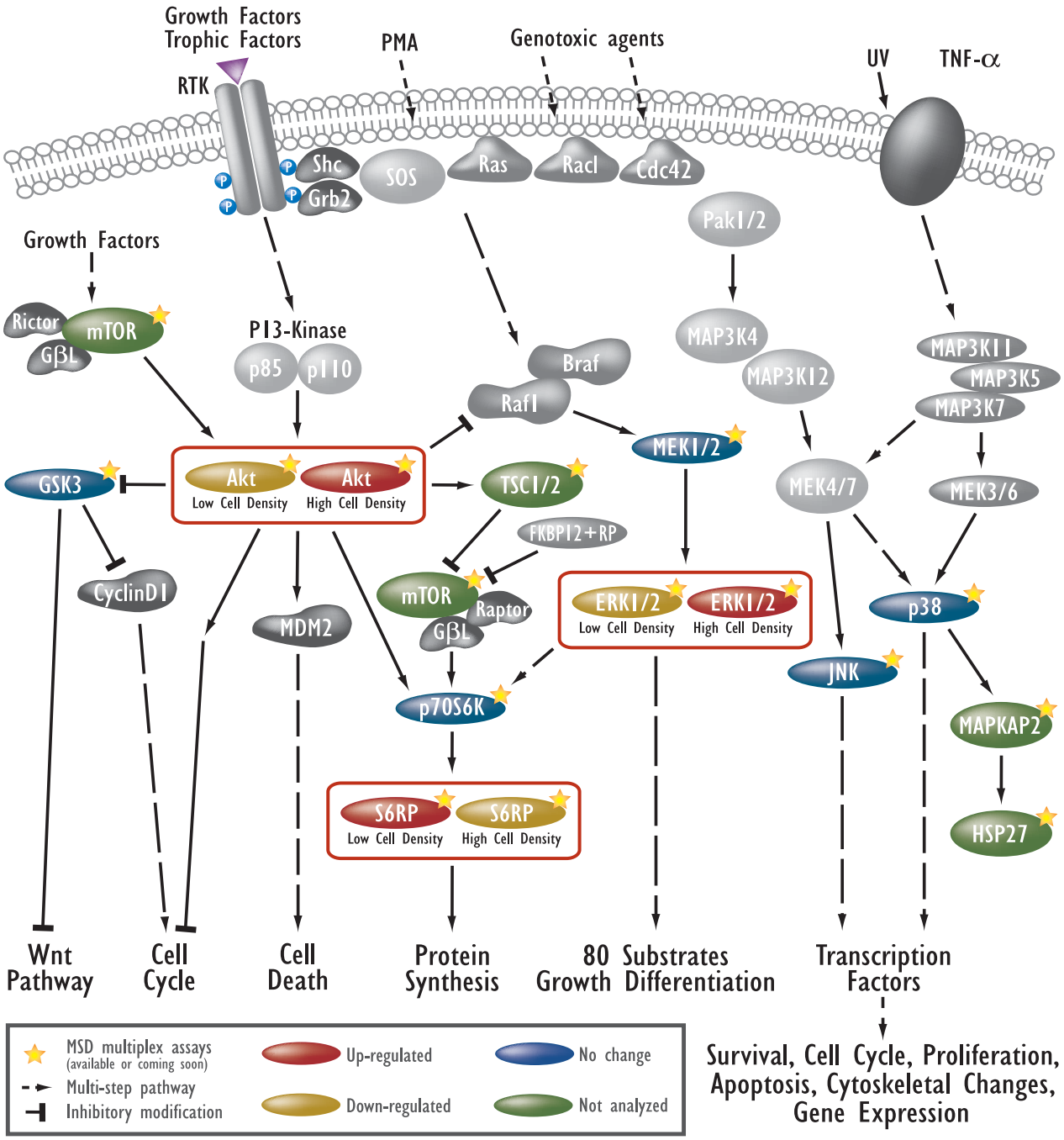


1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 100 µL of Blocker-A for 1 hr and washed with TBST.
2. 25 µL of cell lysates are added to the wells and incubated for 1-3 hr with shaking, followed by washing with TBST.
3. 25 µL MSD SULFO-TAG™ labeled antibodies (in antibody dilution buffer) are added to the wells and incubated for 1 hr with shaking, followed by washing with TBST.
4. 150 µL Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR™ 6000 instrument.

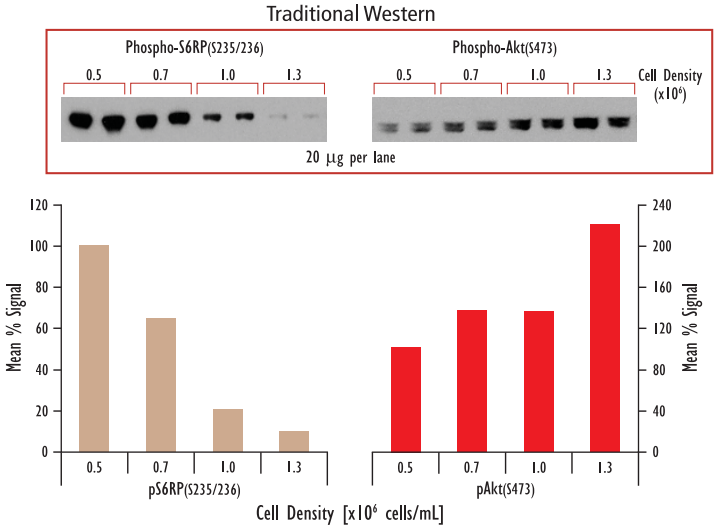
Cell Culture

Jurkat cells were seeded at various cell densities and grown 20 hours to achieve cell densities of 0.5, 0.7, 1.0 and 1.3 x 10^6 cells / mL. Whole cell lysates were prepared in MSD Tris Lysis Buffer and stored frozen until further analysis. Whole cell lysates were either added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibody) and analyzed in multiplexed sandwich assays, or analyzed by traditional western blots.

Modulators of Phosphoprotein Accumulation in Jurkat Cells Including Cell Density



Multiplexed Phospho-S6RP(S235/236), Phospho-Akt(S473) Assay Reveals Reciprocal Regulation of Constitutive Phosphorylation as a Function of Cell Density

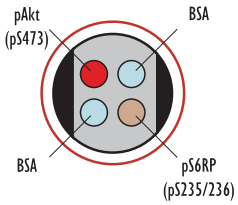


		Jurkat Cell Density [cells/mL]							
	Lysate (µg)	0.5x10⁶		0.7x10⁶		1x10⁶		1.3x10⁶	
		Average	%CV	Average	%CV	Average	%CV	Average	%CV
pS6RP (S235/236)	0.05	1,574	10	964	5	471	7	331	3
	0.2	4,955	2	2,998	4	978	20	481	7
	0.8	13,270	3	9,770	22	2,966	10	890	13
	3	28,660	5	17,361	1	3,777	2	1,894	35
	13	36,340	5	23,863	1	5,927	3	1,799	12
	Ave%	100		64		20		10	
pAkt (S473)	0.05	141	5	136	3	149	4	201	6
	0.2	390	15	485	16	415	29	783	4
	0.8	1,532	1	2,307	10	2,337	3	4,057	11
	3	9,077	3	13,548	8	14,135	10	21,826	7
	13	40,373	3	60,989	1	59,391	2	94,018	7
	Ave%	100		134		133		216	

Regulation was evident at all levels of lysate input. Bar graphs were derived from a mean of the lysate concentrations examined with the highest signal set at 100%.

The sensitivity of the multiplex assay allows characterization with as little as 50 ng cell lysate per well.

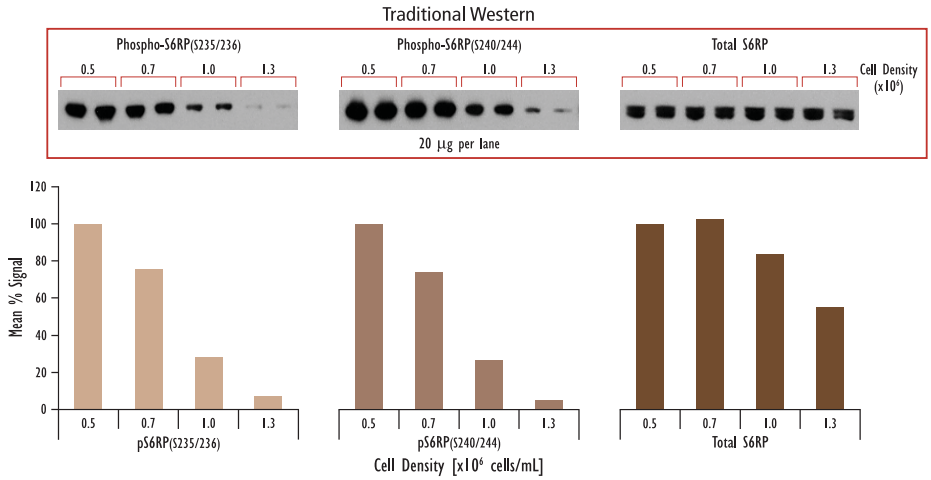
Inhibition of Constitutive Phosphorylation by Rapamycin



	Lysate (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pAkt (S473)	0			55	15	
	0.2	799	6	60	9	**13.3**
	0.8	4,536	6	60	9	**75.6**
	3	22,840	1	138	12	**166.1**
pS6RP (S235/236)	0			287	4	
	0.2	3,565	5	273	8	**13.1**
	0.8	12,253	25	250	0	**49.0**
	3	20,008	5	226	1	**88.5**

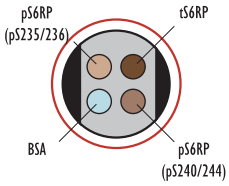
Control Lysates = Logarithmically growing Jurkat cells were treated with rapamycin (1 µM; 3 hr)(negative), or calyculin A (50 nM; 30 min) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibodies) and analyzed in a multiplexed sandwich assay. Data shown for the control lysates was obtained using whole cell lysates prepared from Jurkat cells at a fixed density of 1x10⁶/mL.

An Ultra-Sensitive Multiplex S6RP Assay Shows that (S235/236) and (S240/244) are Both Regulated in a Cell Density Dependent Manner



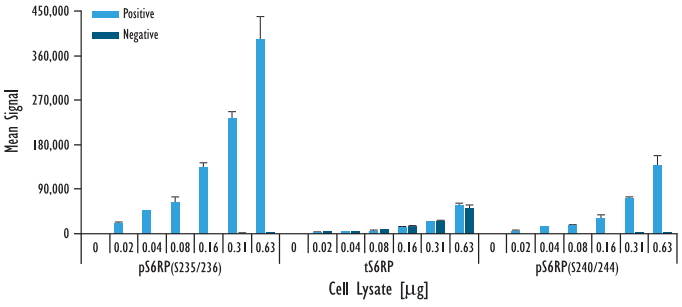
Regulation was evident at all levels of lysate input. Bar graphs were derived from a mean of the lysate concentrations examined with the highest signal set at 100%.

The sensitivity of this multiplex assay allows characterization at sub-microgram levels of cell lysate input per well.



		Jurkat Cell Density [cells/mL]							
	Lysate (µg)	0.5x10⁶		0.7x10⁶		1x10⁶		1.3x10⁶	
		Average	%CV	Average	%CV	Average	%CV	Average	%CV
pS6RP (S235/236)	0.63	135,952	3	102,032	3	32,748	6	7,357	0
	1.25	255,019	29	173,349	5	66,826	18	16,465	19
	2.5	333,071	4	272,530	1	109,271	14	23,643	11
	5	509,880	1	396,227	3	144,357	2	44,219	25
	Ave%	100		76		28		7	
pS6RP (S240/244)	0.63	76,303	5	53,291	1	15,368	10	2,889	5
	1.25	159,024	39	102,653	9	34,966	18	5,799	38
	2.5	202,989	0	174,167	9	63,096	20	10,011	28
	5	290,130	2	219,835	3	98,763	0	23,886	43
	Ave%	100		74		27		5	
tS6RP	0.63	50,041	1	50,320	7	42,126	2	28,914	4
	1.25	112,228	32	97,519	9	92,074	14	51,829	19
	2.5	150,063	4	172,720	8	131,673	5	74,667	1
	5	257,688	2	277,541	0	206,872	2	172,478	42
	Ave%	100		103		84		55	

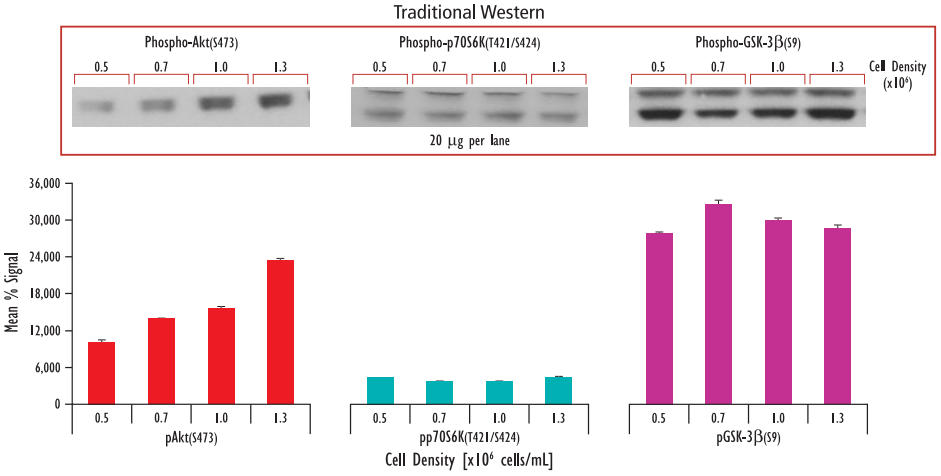
Two Phosphorylation Sites on S6RP Remain Sensitive to Rapamycin Inhibition at a Fixed Jurkat Cell Density



	Lysate (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pS6RP (S235/236)	0			486	13	
	0.2	21,281	12	176	8	120.9
	0.04	46,773	3	255	3	183.8
	0.08	63,382	18	285	1	222.8
	0.16	134,691	6	376	4	358.7
	0.31	233,409	6	807	17	289.2
	0.63	393,700	11	1,429	16	275.5
tS6RP	0			430	12	
	0.2	2,570	25	2,735	9	0.9
	0.04	5,744	9	5,315	1	1.1
	0.08	6,185	7	8,203	15	0.8
	0.16	13,232	9	14,309	10	0.9
	0.31	25,138	1	24,953	11	1.0
	0.63	56,823	8	49,982	15	1.1
pS6RP (S240/244)	0			427	12	
	0.2	5,632	17	156	1	36.2
	0.04	13,865	2	272	5	51.1
	0.08	16,441	15	323	16	50.9
	0.16	30,838	25	498	1	61.9
	0.31	70,388	7	1,014	2	69.4
	0.63	138,752	14	1,633	17	85.0

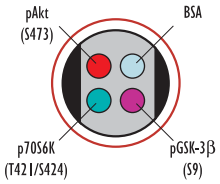
Logarithmically growing Jurkat cells were treated with rapamycin (1 µM; 3 hr)(negative), or PMA (200 nM) + calyculin A (10 nM; 30 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibodies) and analyzed in a multiplexed sandwich assay. Data was obtained using whole cell lysates prepared from Jurkat cells at a fixed density of 1x10⁶/mL.

Additional Downstream Targets of Akt are not Regulated as a Function of Cell Density



Bar graphs shown above derived from an MSD multiplex assay in which pAkt(S473), pp70S6K(T421/S424) and pGSK-3β(S9) were monitored simultaneously in the same well. The amount of cell lysate remained fixed at 20 µg per well.

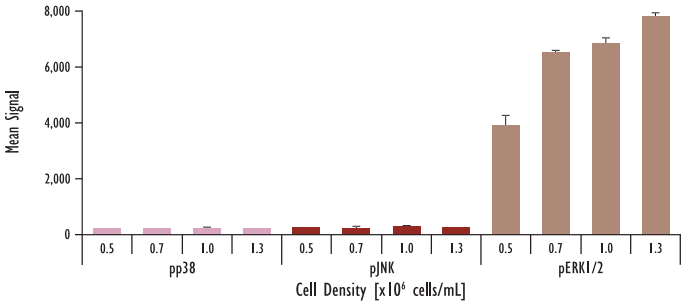
A Multiplex Assay Quantifies Inhibition of pAkt and pp70S6K by LY294002 and pGSK-3β by Staurosporine



	Lysate (µg)	Positive Average	Positive %CV	Negative Average	Negative %CV	P/N
pAkt (S473)	0			141	12	
	1.25	1,441	9	207	3	**7.0**
	5	5,469	6	284	15	**19.3**
	20	25,627	6	466	1	**55.1**
pp70S6K (T421/S424)	0			323	2	
	1.25	1,433	5	356	8	**4.0**
	5	4,325	1	417	4	**10.4**
	20	11,652	1	734	0	**15.9**
pGSK-3β (S9)	0			230	0	
	1.25	4,157	2	443	4	**9.4**
	5	9,752	0	891	6	**11.0**
	20	23,784	6	2,149	4	**11.1**

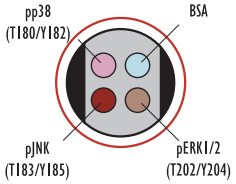
Logarithmically growing Jurkat cells were treated with LY294002 + staurosporine (negative), or PMA (200 nM; 30 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibodies) and analyzed in a multiplexed sandwich assay. Data shown for the control lysates was obtained using whole cell lysates prepared from Jurkat cells at a fixed density of $1x10^6$/mL.

Multiplexed MAPK-Signaling Panel Reveals Phosphorylated ERK1/2 is Regulated by Jurkat Cell Density



Bar graphs shown above derived from a multiplex MAPK assay in which pp38, pJNK and pERK1/2 were monitored simultaneously in the same well. The amount of cell lysate remained fixed at 2.5 µg per well. Constitutive phosphorylation was monitored in logarithmically growing cells at various densities.

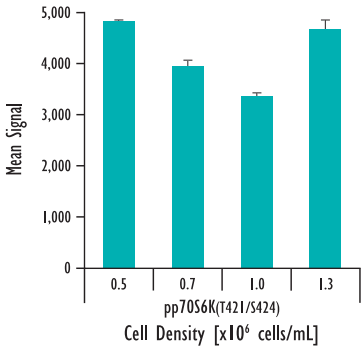
The MSD MAPK Multiplex Assay Quantifies Phosphoproteins at Sub-microgram Levels of HEK293 Whole Cell Lysate

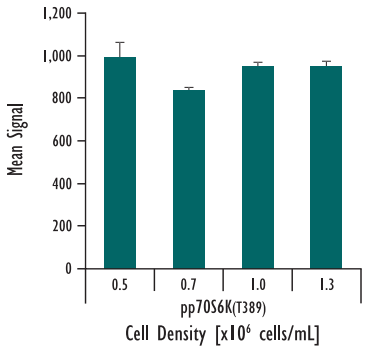


	Lysate (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp38 (T180/Y182)	0			145	16	
	0.63	1,070	0	324	12	3.3
	1.25	2,147	1	327	1	6.6
	2.5	4,513	7	255	7	17.7
	5	10,763	8	225	3	47.8
	10	35,186	9	181	0	194.4
pJNK (T183/Y185)	0			303	4	
	0.31	1,608	4	412	1	3.9
	0.63	2,427	7	507	4	4.8
	1.25	4,303	2	554	0	7.8
	2.5	7,582	7	575	2	13.2
	5	10,187	14	640	2	15.9
	10	15,167	1	731	0	20.8
pERK1/2 (T202/Y204)	0			193	4	
	0.08	2,569	5	213	5	12.1
	0.16	4,857	2	236	1	20.6
	0.31	7,694	2	283	1	27.2
	0.63	9,391	3	356	4	26.4
	1.25	8,828	4	347	1	25.4
	2.5	10,067	6	297	4	34.0

Growing HEK293 cells were treated with rapamycin (1 µM; 3 hr)(negative), or UV + calyculin A (50 nM; 30 min) (positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibodies) and analyzed in a multiplexed sandwich assay.

Both T421/S424 and T389 Phosphorylation Sites on p70S6K are Not Regulated by Jurkat Cell Density





Bar graphs shown above were derived from MSD multiplex pp70S6K assays in which the amount of cell lysate remained fixed at 20 µg per well. Constitutive phosphorylation was monitored in logarithmically growing cells at various densities.

Constitutive Phosphorylation of p70S6K at T421/S424 and T389 is inhibited by Rapamycin at a Fixed Cell Density

	Lysate (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp70S6K (T421/S424)	0			158	6	
	0.08	732	6	208	15	**3.5**
	0.16	1,347	3	236	3	**5.7**
	0.3	2,715	4	273	11	**10.0**
	0.6	4,702	5	336	13	**14.0**
	1.3	9,008	1	547	6	**16.5**
	2.5	17,067	1	810	1	**21.1**
	5	29,978	1	1,269	9	**23.6**
	10	56,732	4	1,995	2	**28.4**
	20	112,924	2	3,449	4	**32.7**

	Lysate (µg)	Positive		Negative		P/N
		Average	%CV	Average	%CV	
pp70S6K (T389)	0			90	6	
	0.08	965	5	110	2	**8.8**
	0.16	1,989	1	127	3	**15.7**
	0.3	3,792	0	184	8	**20.6**
	0.6	6,768	1	244	5	**27.7**
	1.3	12,499	2	305	2	**41.0**
	2.5	16,723	1	297	2	**56.4**
	5	24,503	2	287	6	**85.4**
	10	34,373	1	323	7	**106.6**
	20	41,304	1	651	0	**63.4**

Growing HEK293 cells were treated with rapamycin (1 µM; 3 hr)(negative), or calyculin A (50 nM; 30 min)(positive). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot plates (pre-coated with capture antibodies) and analyzed in a multiplexed sandwich assay.

Conclusions

1. We show reciprocal regulation of the constitutive phosphorylation of Akt and S6RP in mitotically growing Jurkat cells, where low cell density correlates with maximal phosphorylation of S6RP, while high cell density correlates with maximum phosphorylation of Akt.

2. The cell density dependent regulation described here does not fit a simple model for regulation in the Akt and MAPK pathways (panel 3). We conclude that there are additional regulators of the phosphoproteins studied here that account for the cell density dependent effects on phosphorylation.

3. ERK1/2 exhibits regulation similar to Akt being maximally phosphorylated at high cell density.

4. The effect of cell density was studied using MSD multiplex assays where MAPK and Akt pathway phosphoproteins were monitored simultaneously in the same well using whole cell lysates.

5. The data shown reveal highly sensitive assays obtained at a fixed Jurkat cell density. The sub-microgram levels of lysate input makes these multiplex assays easily amenable to 384-well cell culture applications.