Validation & Assay Performance Summary

CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa Cell Line

Cat. no. K1468

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in a screening application. The following information illustrates the high level of assay testing completed and the validation of assay performance under optimized conditions.

Pathway Description

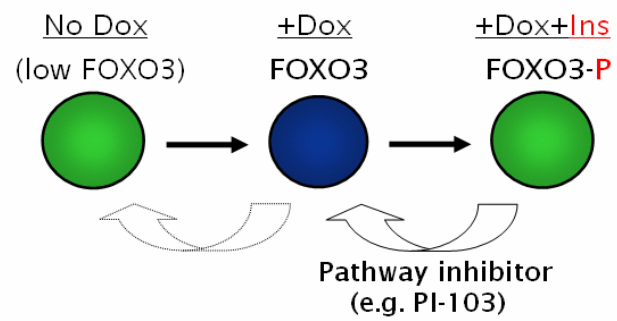
The phosphatidylinositol-3-kinase (PI3K) signaling cascade is essential for cell growth and survival. A series of recent studies indicate that the various PI3K pathway components are targeted by amplification, mutation, and translocation more frequently than any other pathway in cancer patients. The mammalian FOXO subfamily of Forkhead transcription factors is among the best characterized targets of PI3K signaling and consists of three members: FOXO1 (FKHR), FOXO3 (FKHRL1), and FOXO4 (AFX). Growth factor-induced activation of PI3K results in increased downstream activity of the serine/threonine kinase AKT, which in turn phosphorylates the FOXO family of transcription factors and other targets to promote cell survival and oppose apoptosis. Under stress conditions that activate FOXOs (e.g. serum-starvation), they translocate from the cytoplasm to the nucleus where they induce or repress transcription of sets of target genes. FOXO3, for example, can function as a trigger for apoptosis through transcription of cell death genes (e.g. FASL, BIM).

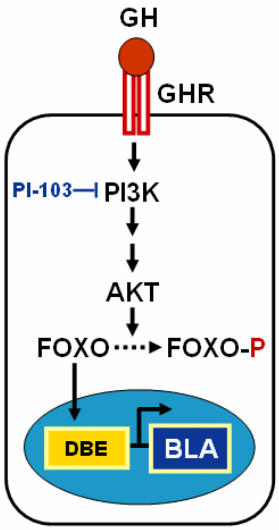
Cell Line Description

CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa was engineered by lentiviral transduction of HeLa cervical cancer cells with a FOXO3-response element driving beta-lactamase expression (DBE-*bla*) along with tetracycline repressor and tetracycline-inducible FOXO3 constructs. This cell line is a clonal population isolated by flow cytometry and has been tested for robust assay performance by assessing a variety of assay parameters. Addition of doxycycline, a tetracycline analog, to these cells allows for FOXO3 transcription factor expression and subsequent beta-lactamase expression. Growth hormone (e.g. insulin or IGF1) activation of the PI3K/AKT signaling pathway leads to phosphorylation and inactivation of FOXO3 and concomitant suppression of beta-lactamase expression. Application of pathway inhibitors (e.g. PI-103) restores FOXO3 activity and beta-lactamase expression.

CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa features:

- Tetracycline/doxycycline-inducible FOXO3 expression
- FOXO3 response element
- GeneBLAzer® beta-lactamase reporter gene readout





Validation Summary

Testing and validation of this assay was evaluated in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions (n=3)**

Average Insulin EC_{50}	= 5.0 nM
Average Z'-Factor (EC_{100})	= >0.5
Average Response Ratio	= 3.7
Recommended cells/well	= 10,000
Recommended [DMSO]	= up to 1 %
Stimulation Time	= 16-20 hours
Max. [Stimulation]	= 1000 nM insulin

2. **Alternate ligand dose response**

IGF1 EC_{50}	= 1.6 nM
Insulin (IVGN) EC_{50}	= 5.2 nM
Insulin (Sigma) EC_{50}	= 7.1 nM
Dexamethasone EC_{50}	= 5.7 nM
PDGF EC_{50}	= 21 nM

3. **Inhibitor panel**

AKT VIII IC_{50}	= 240 nM
LY294002 IC_{50}	= 810 nM
PI3Ka IV IC_{50}	= 330 nM
PI-103 IC_{50}	= 110 nM
Wortmannin IC_{50}	= 31 nM

4. **Target validation with RNAi**

5. **Assay performance with cryopreserved cells**

6. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

7. **Assay performance with variable cell number**

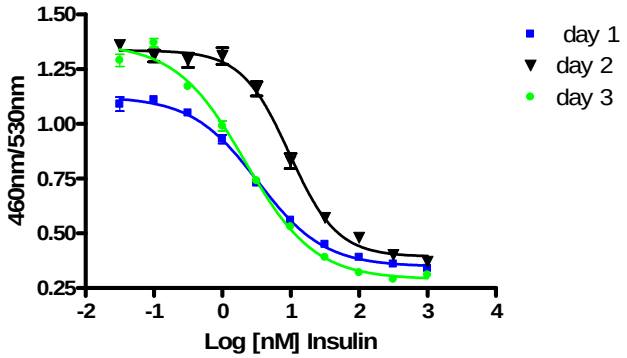
8. **Assay performance with variable DMSO concentration**

9. **Assay performance with variable stimulation time**

10. **Assay performance with variable substrate loading time**

Primary Agonist Dose Response

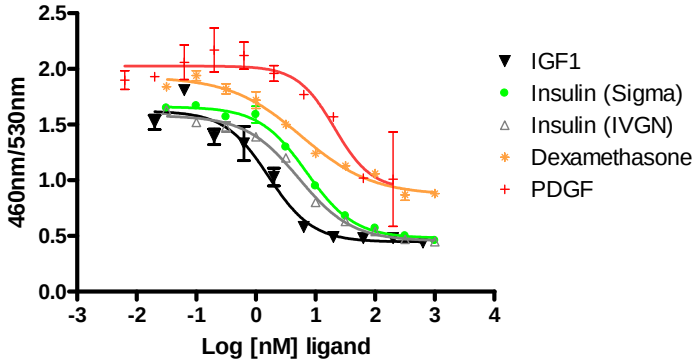
Figure 1 — Insulin dose-response under optimized conditions



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed on three separate days. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. The cells were stimulated overnight with a serial dilution of insulin in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 4 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted for each treatment (n = 16 for each data point).

Alternate Ligand Dose Response

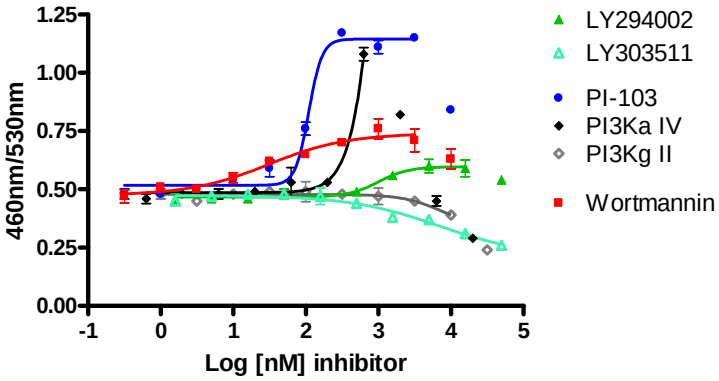
Figure 2 — Ligand dose-response curves



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for response to various ligands. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. The cells were stimulated overnight with serial dilutions of the ligands in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted for each treatment (n = 4 for each data point, except n =2 for PDGF data points).

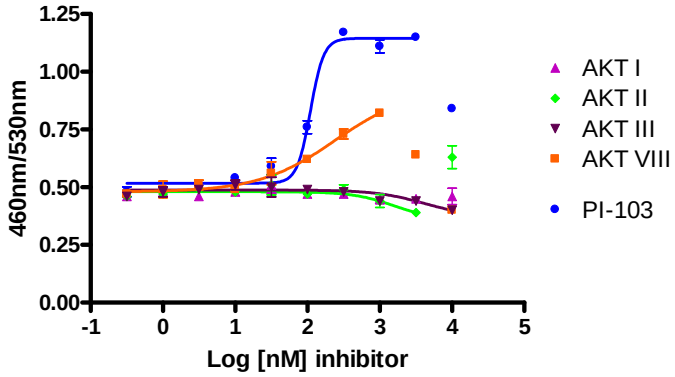
Inhibitor Dose Response

Figure 3A — PI3K inhibitor panel



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for response to various PI3K inhibitors. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. Serial dilutions of the indicated inhibitors were applied to the wells for 30 minutes prior to addition of insulin (100 nM final). Cells were further incubated overnight prior to loading the cells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 2 for each data point).

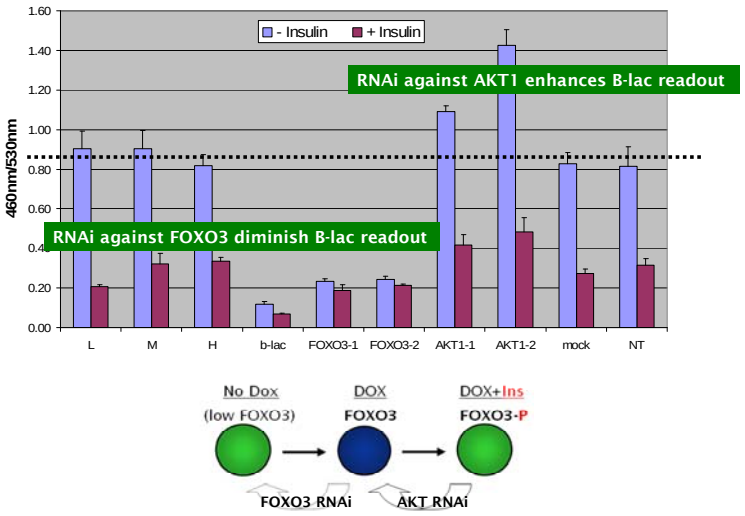
Figure 3B — Additional PI3K/AKT inhibitor panel



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for response to various AKT inhibitors in comparison to PI3K inhibitor PI-103. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. Serial dilutions of the indicated inhibitors were applied to the wells for 30 minutes prior to addition of insulin (100 nM final). Cells were further incubated overnight prior to loading the cells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 2 for each data point).

Target validation with RNAi

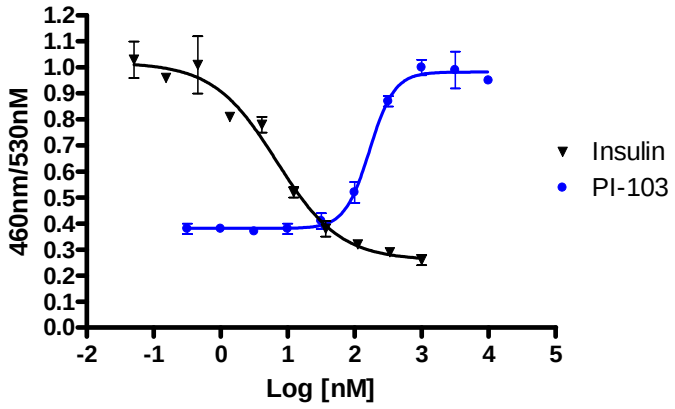
Figure 4 — RNAi knockdown of FOXO3 and AKT



T-REx™ FOXO3 DBE-*bla* HeLa cells were plated in 384-well format at 2500 cells/well and reverse-transfected using Lipofectamine™ RNAiMAX Transfection Reagent and 20 nM of the following panel of RNAi duplexes or control conditions: L (Stealth™ RNAi Negative Control LO GC), M (Stealth™ RNAi Negative Control Med GC), H (Stealth™ RNAi Negative Control Hi GC), b-lac (Beta-lactamase RNAi positive control duplex), FOXO3-1 (FOXO3A Validated Stealth™ DuoPak duplex #1), FOXO3-2 (FOXO3A Validated Stealth™ DuoPak duplex #2), AKT-1 (AKT1 Validated Stealth™ DuoPack duplex #1), AKT-2 (AKT1 Validated Stealth™ DuoPack duplex #2), mock transfected (no RNAi duplex), or NT (non-transfected control). A fixed concentration of Dox (0.5 ug/ml final) was added to the cells which were then incubated for 40 hours before the transfection media was aspirated from the wells and replaced with serum-free media containing 0.5 ug/ml Dox and +/- 316 nM final concentration of insulin. Cells were incubated in serum-free conditions for 6.5 hours prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) plus probenecid for 5 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted for each treatment (n = 4 for each data point).

Assay performance with cryopreserved cells

Figure 5 – Assay performance with cryopreserved cells



T-REx™ FOXO3 DBE-*bla* HeLa cells were tested for assay performance following plating of cryopreserved cells. Doxycycline (5 ng/ml) was added to a carrier flask of cells for ~24 h prior to freezing down the cells. At the time of assay setup, a vial of cryopreserved cells was thawed and immediately used to seed a 384-well assay plate in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. Serial dilutions of PI-103 inhibitor or vehicle (0.1 % final DMSO) were applied to the wells for 30 minutes prior to addition of insulin (100 nM final to PI-103 inhibitor wells or separate serial dilution). Cells were further incubated overnight prior to loading the cells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 2 for each data point).

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection (Blasticidin, Hygromycin, or Zeocin) and culture them in Growth Medium with selection. Pass or feed cells 2-3 times a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 10% and 90% confluence.

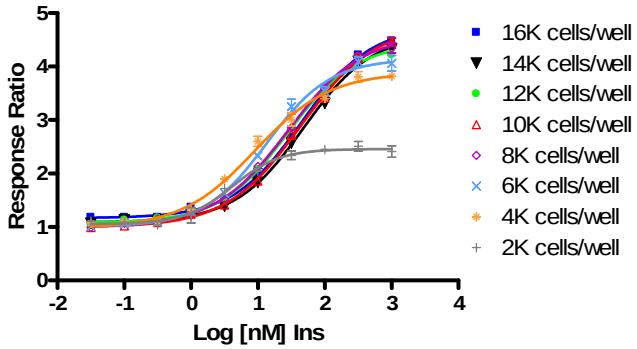
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
DMEM with GlutaMAX™	500 mL	500 mL	500 mL	—
Dialyzed FBS (dFBS) **Do not substitute!**	50 mL	50 mL	50 mL	—
HEPES (1 M)	12.5 mL	12.5 mL	12.5 mL	
NEAA (100x)	5 mL	5 mL	5 mL	—
Pen/Strep (100x)	5 mL	5 mL	5 mL	—
Blasticidin	—	5 µg/mL	—	—
Hygromycin B	—	125 µg/mL	—	—
Zeocin	—	75 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

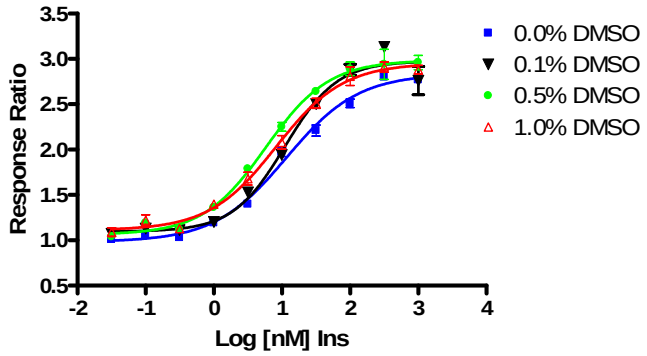
Figure 6— Insulin dose response with varying cell plating density



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for insulin dose response while varying cell plating density. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at the indicated cell densities. The cells were stimulated overnight with a serial dilution of insulin in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratios (460/530 ratio of the untreated control divided by the 460/530 ratios of the insulin treated points) were plotted (n = 4 for each data point).

Assay Performance with variable DMSO concentration

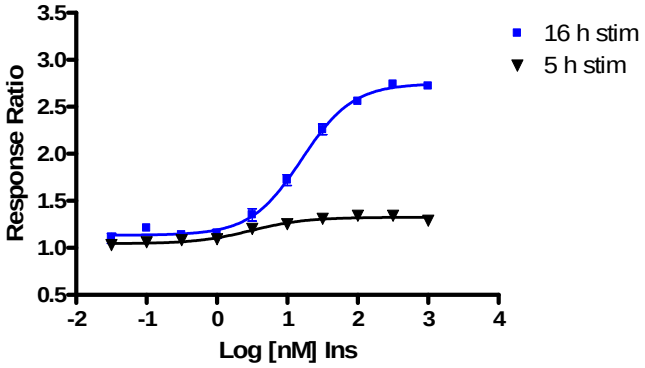
Figure 7 – Insulin dose response with 0, 0.1, 0.5 and 1% DMSO.



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for insulin dose response with variable final DMSO concentration. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. The cells were stimulated overnight with a serial dilution of insulin in the presence of varying DMSO concentrations prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratios (460/530 ratio of the untreated control divided by the 460/530 ratios of the insulin treated points) were plotted (n = 8 for each data point).

Assay performance with Variable Stimulation Time

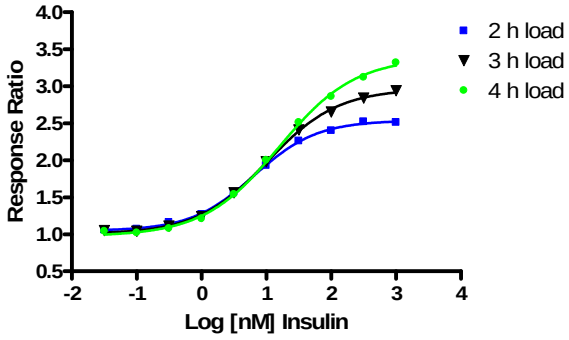
Figure 8 – Insulin dose response with 5 and 16 hour stimulation times



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for the effect of insulin stimulation time. Doxycycline (5 ng/ml) was added to carrier flasks of cells in the morning and in the afternoon (for 16 h stim time) or the morning of the next day (for 5 h stim time) the cells were plated in 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. The cells were stimulated with a serial dilution of insulin in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratios (460/530 ratio of the untreated control divided by the 460/530 ratios of the insulin treated points) were plotted (n = 8 for each data point).

Assay performance with Variable Substrate Loading Time

Figure 9 – Insulin dose response with 2, 3, and 4 hour loading times



T-REx™ FOXO3 DBE-*bla* HeLa cells were assayed for the effect of substrate loading time. Doxycycline (5 ng/ml) was added to the carrier flask of cells in the morning. In the afternoon, the cells were plated into 384-well format in low serum-containing Assay Medium supplemented with 5 ng/ml doxycycline at 10,000 cells/well. The cells were stimulated overnight with a serial dilution of insulin in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for the indicated times. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratios (460/530 ratio of the untreated control divided by the 460/530 ratios of the insulin treated points) were plotted (n = 16 for each data point).

References

1. Hennessy BT, Smith DL, Ram PT, Lu Y, and Mills GB. (2005) **Exploiting the PI3K/AKT pathway for cancer drug discovery**. *Nature Reviews Drug Discovery* 4:988-1004.

2. Birkenkamp KU and Coffer PJ. (2003) **Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors**. *Biochemical Society Transactions* 31:292-297.

3. Accili D and Arden KC. (2004) **FoxOs at the crossroads of cellular metabolism, differentiation, and transformation**. *Cell* 117:421-426.

4. Fan Q-W, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D, Shokat KM, and Weiss WA. (2006) **A dual PI3 Kinase/mTOR inhibitor reveals emergent efficacy in glioma**. *Cancer Cell* 9:341-349.