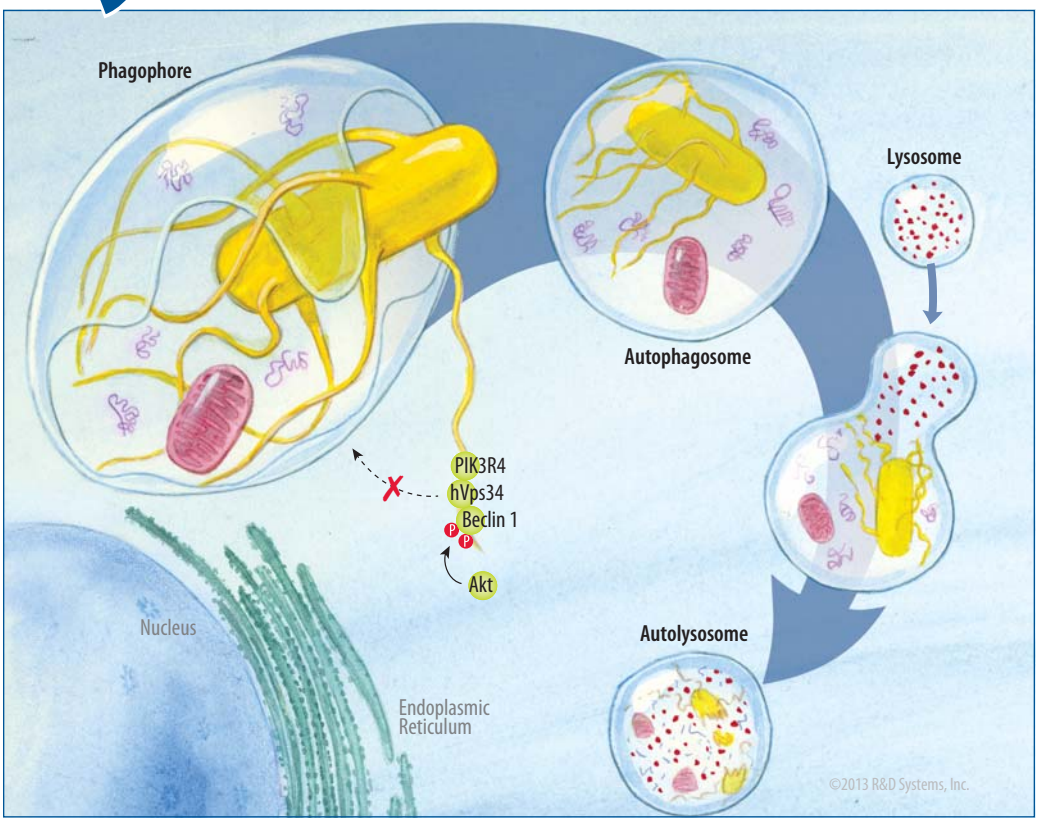
Special Cancer Edition!

Cytokine BULLETIN

R&D Systems Cytokine Bulletin
2013 | Cancer Edition

INSIDE



Akt Directly Phosphorylates Beclin 1 to Suppress Autophagy. Autophagy is a process by which cells degrade long-lived proteins, organelles, and certain types of bacteria in the cytoplasm. Briefly, the Beclin 1 complex is required for the formation of phagophores that subsequently envelope cytoplasmic components, form an autophagosome, and fuse with a lysosome. The cytoplasmic components are then degraded within the resulting autolysosome. Akt directly phosphorylates Beclin 1, which inhibits Beclin 1 complex activity and suppresses autophagy. The article below details this novel mechanism of autophagy suppression and highlights the implications for cancer.

A Novel Pathway for TOR-independent Autophagy Regulation by Akt

Autophagy, which degrades cytoplasmic components to generate recycled nutrients, can have either an oncogenic or tumor suppressive role in cancer.[1] In contrast, Akt is known to promote cellular transformation and tumorigenesis, and dysregulated Akt activity is observed in many cancer types.[2] The oncogenic potential of dysregulated Akt is often associated with its role in the positive regulation of cellular growth, proliferation, and survival. However, Akt signaling is also known to suppress autophagy.[3] Akt negatively regulates autophagy in response to mitogens via activation of Target of Rapamycin (TOR), which inhibits multiple autophagy-promoting proteins via phosphorylation.[4] It has also been reported that Akt can regulate autophagy in a TOR-independent manner, but the mechanism has remained elusive.[5] A recent paper by Wang *et al.* describes a TOR-independent mechanism by which Akt can suppress autophagy. This mechanism could be relevant for the development of future cancer therapies.[6]

To confirm that Akt activity could suppress autophagy independent of TOR activity, a constitutively active, myristoylated form of Akt1 (myr-Akt) was utilized. In the presence of Torin1, a TOR inhibitor, myr-Akt expression resulted in stabilized p62 and a reduced number of LC3 puncta in HeLa cells, both of which are indicative of reduced autophagy.[6-8] The observation that TOR activity was dispensable for autophagy suppression suggested that Akt might directly target an autophagy protein. Previous studies and immunoprecipitation experiments indicated that Akt can interact with Beclin 1, a core autophagy protein.[6,9] Recombinant human Akt1 was also able to directly phosphorylate Beclin 1 in an *in vitro* kinase assay.[6] In myr-Akt-expressing HeLa cells, Beclin 1 was phosphorylated at Ser234 and Ser295 in the presence of Torin1. These results suggest that Akt interacts with, and phosphorylates, Beclin 1 in a manner that is independent of TOR activity.

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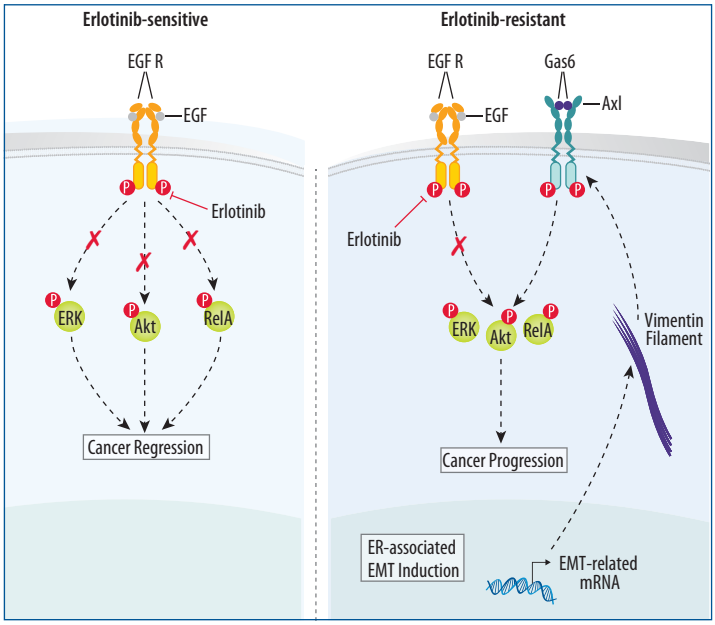
Axl Overexpression Promotes TKI Resistance in Non-Small Cell Lung Cancer

Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for approximately 80% of diagnosed cases.[1] Epidermal Growth Factor Receptor (EGF R), a receptor tyrosine kinase (RTK), is dysregulated in 40-80% of NSCLC cancers, and numerous mutations that activate EGF R have been detected in primary NSCLC tumors.[2,3] Therefore, tyrosine kinase inhibitors (TKIs) that target EGF R are commonly used for lung cancer therapy. Erlotinib is a TKI that was approved by the FDA in 2004 for NSCLC patients and has been shown to be effective in those harboring EGF R mutations.[4,5] Unfortunately, NSCLC patients develop erlotinib resistance (ER) within 10-14 months of primary treatment.[6] In a recent paper, Zhang *et al.* identified a novel mechanism for ER that could lead to the development of new drug targets for NSCLC patients that have acquired ER.[7]

To identify candidate genes involved in the development of ER, mRNA expression profiles from cell culture and mouse xenograft models of acquired ER were compared to erlotinib-sensitive controls.[7] This analysis revealed that the mRNA levels of *AXL*, an RTK, were significantly higher in both ER HCC827 human NSCLC cell lines and 15 of 17 ER tumors. Total and phospho-Axl protein levels were also increased in the ER cell lines and tumors. To determine if *AXL* overexpression had a causative role in ER acquisition, ER cell lines and tumors from the mouse xenograft model of acquired ER were treated with small interfering (si) RNA or short hairpin (sh) RNA, respectively. Sensitivity to erlotinib was restored in both cases. These data suggest that *AXL* overexpression is necessary for the acquisition of ER in these cell culture and *in vivo* models.

To determine if Axl kinase activity was required in this model of ER, Zhang *et al.* transiently overexpressed either wild-type Axl or a kinase dead Axl mutant in HCC827 cells.[7] Only wild-type Axl was able to induce ER, suggesting that its kinase activity was required for the development of ER. Axl has been reported to activate MAPK, Akt, and NFκB signaling in cancer cell lines.[8,9] Treatment of ER cells with erlotinib, along with either *AXL*-specific siRNA or Axl inhibitors, reduced the phosphorylation of ERK, Akt, and RelA, whereas treatment with erlotinib alone did not.[7] These results suggested that the MAPK, Akt, and NFκB pathways may mediate the acquisition of ER downstream of Axl kinase activity.



WEB EXCLUSIVE

To view Axl detection tools offered by R&D Systems, including the total and phospho-Axl antibodies used in Zhang *et al.*, please visit: www.RnDSystems.com/Axl

Epithelial to mesenchymal transition (EMT), the process by which cells lose epithelial characteristics and acquire a migratory, mesenchymal phenotype, has been linked to the development of ER.[10] Since Axl can be induced during EMT, this process might contribute to Axl-dependent ER acquisition.[11] Interestingly, the ER xenograft-derived tumors and ER cell lines expressed several biomarkers of EMT, including Vimentin upregulation.[7,12] Additionally, siRNA knockdown experiments showed that Vimentin was required for Axl overexpression and ER acquisition in HCC827 cells.[7] These data suggest that EMT may have a role in the development of Axl-dependent ER in this cell culture model.

To determine if Axl-dependent ER might be clinically relevant, the authors asked if Axl overexpression could be detected in NSCLC patients with acquired ER.[7] Axl expression was assessed by immunohistochemistry in 35 EGF R-mutant NSCLC specimens from individuals before treatment with erlotinib or gefitinib, another TKI, and then after acquisition of TKI resistance. Axl expression levels were at least two-fold higher in seven out of 35 TKI-resistant specimens compared to pre-treatment specimens. This suggested that overexpression of Axl may be important for ER acquisition in some NSCLC patients.

This new study utilized both cell culture and mouse xenograft models to identify a novel Axl-dependent mechanism by which EGF R-mutant NSCLC can acquire resistance to TKIs. It is likely complex, potentially involving several downstream signaling pathways and an association with EMT. Axl overexpression observed in tumor tissue from patients with TKI-resistant NSCLC supported their model and suggested that this mechanism could have clinical relevance. It is possible that future therapies combining Axl inhibition with TKI treatment could delay or prevent the development of TKI resistance.

References

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This symbol denotes references that cite the use of R&D Systems products.

Axl Overexpression is a Novel Mechanism of TKI Resistance in EGF R-mutant NSCLC. EGF R is a receptor tyrosine kinase (RTK) that is often mutationally activated in non-small cell lung cancer (NSCLC). Its activity can result in the activation of MAPK (ERK), Akt, and NFκB (RelA) signaling and promote cancer progression. Erlotinib treatment can block the activity of mutant EGF R and its downstream signaling pathways, resulting in cancer regression (left panel). Zhang *et al.* show that *in vitro* and *in vivo* models of NSCLC with acquired erlotinib resistance overexpress Axl, another RTK, which reactivates the MAPK, Akt, and NFκB signaling pathways, and promotes cancer progression in the presence of erlotinib (right panel). Furthermore, Axl overexpression was dependent on the expression of Vimentin, a marker for EMT, suggesting that EMT may play a role in the acquisition of resistance to erlotinib.

Non-traditional Notch Activation Promotes the Cancer Stem Cell Phenotype

Cancer stem cells (CSCs) are classically described as a tumor cell subpopulation that is multipotent, oncogenic, and capable of self-renewal.[1,2] They may serve as an ongoing source for differentiating tumor cells and can exhibit characteristics that confer resistance to chemotherapy, including low proliferation rates and the expression of detoxifying enzymes such as aldehyde dehydrogenase (ALDH). These features place CSCs in a position to sustain tumors over the long-term, and understanding how these cells are regulated is crucial if they are to be targeted for therapy. Cells with characteristics of CSCs have been described in a range of tumor types. This includes colorectal cancer (CRC), the world's third most prevalent tumor subtype accounting for almost 10% of all cancer diagnoses.[3] A recent study by Lu *et al.* provides evidence that a non-traditional activation of the Notch pathway can drive human CRC cells toward a CSC phenotype.[4]

WEB EXCLUSIVE

To learn more about Notch signaling visit our new interactive Notch Pathway: www.RnDSystems.com/Pathways_Notch

Studies have suggested that the tumor vasculature may act as more than a simple conduit that provides nutrients and O_2. Vascular endothelial cells (ECs) might also act as a source for factors that promote tumor growth or the expansion of tumorigenic stem cells.[5] In support of this activity, when cells derived from CRC tumors were cultured in EC-conditioned medium (EC CM), they exhibited several characteristics of CSC-like cells. These included increased expression of the marker CD133, enhanced ALDH activity, greater *in vitro* sphere-forming ability, and resistance to chemotherapeutic agents. These observations might result from either the expansion of existing CSC-like cells or by dedifferentiation. Interestingly, the latter appeared to be true as isolated tumor cells exhibiting characteristics of differentiation could be driven toward the CSC phenotype using EC CM. Supporting the physiological relevance of these *in vitro* observations, CRC cells pre-incubated with EC CM exhibited greater tumorigenicity and metastatic potential in mouse xenografts.

Wnt, Sonic Hedgehog, and Notch pathways are well known for their morphogenic functions during early development. When dysregulated, they can have complex, context-dependent effects on tumorigenesis and many studies have linked morphogen activity to CSCs.[6-9] Therefore, morphogens were investigated as a potential source of the CSC-inducing bioactivity observed in EC CM. Experiments using transcriptional reporters of Wnt, Sonic Hedgehog, and Notch indicated that only Notch activity was increased in CRC cells exposed to EC CM. In addition, tumor cells co-expressing CD133 and the active form of Notch were found adjacent to blood vessels in primary human CRC tumors and liver metastases, supporting a relationship between ECs and Notch activity *in vivo*. Ligands capable of inducing Notch activity include members of the Delta-like (DLL) and Jagged families, and in this model Jagged 1 appeared to be of importance. Immunoreactivity for Jagged 1 was detected in EC CM, and CSC-promoting activity was blocked by suppressing EC-derived Jagged 1 with either small interfering (si) RNA or neutralizing antibodies.

Both Notch and its ligands are transmembrane proteins. In the canonical Notch pathway, ligand binding to receptor involves direct cell/cell interaction. Subsequently, Notch is proteolytically cleaved and its intracellular domain translocates to the nucleus, while the ligand/Notch extracellular domain complex is endocytosed by the ligand-expressing cell.[10] However, the presence of a low molecular weight version of Jagged 1 in EC CM suggested that a soluble, truncated form could be the active factor responsible for promoting CSC features in CRCs. Previous studies have indicated that the membrane-anchored enzyme TACE/ADAM17 can mediate the shedding of transmembrane proteins including Jagged 1.[11] Several lines of evidence indicated that the protease TACE/ADAM17 was important for generating soluble Jagged 1. Suppression of the protease, either pharmacologically or via siRNA, inhibited EC-mediated CSC induction in CRCs. In addition, co-transfection of TACE/ADAM17 and Jagged 1 enabled cultured fibroblasts to produce CSC-inducing conditioned media. Highlighting the importance of this mechanism, tumorigenicity was significantly inhibited by pharmacologically blocking TACE/ADAM17 in a mouse xenograft CRC model.

The CSC hypothesis suggests that resilient, tumorigenic stem cells that are capable of seeding new tumors exist within a cancer cell population. Many recent studies over a range of cancer subtypes suggest that the CSC population may be more heterogeneous and dynamic than originally thought. For instance, multiple genetically distinct CSC clones may exist within a tumor cell population, and certain CSCs might exhibit altered migratory capacity or the ability to fluctuate between states of differentiation depending on the microenvironment.[8,12-15] This new study demonstrates a mechanism by which EC-derived Jagged 1 can drive CRC cells toward a CSC-like phenotype. It involves a soluble form of Jagged 1, and therefore, a non-traditional way of activating the Notch pathway. Notch-activated CRCs exhibited increased tumorigenicity, enhanced metastatic capability, and resistance to chemotherapy.[4] All of these traits promote

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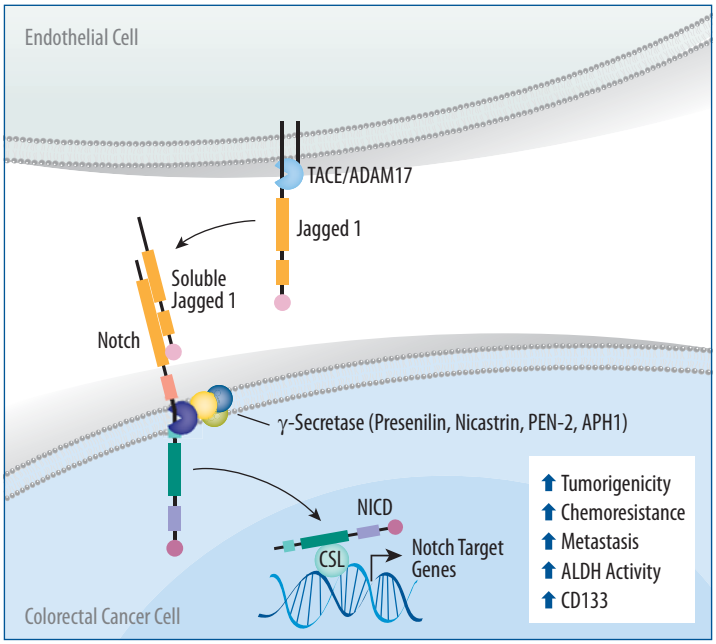
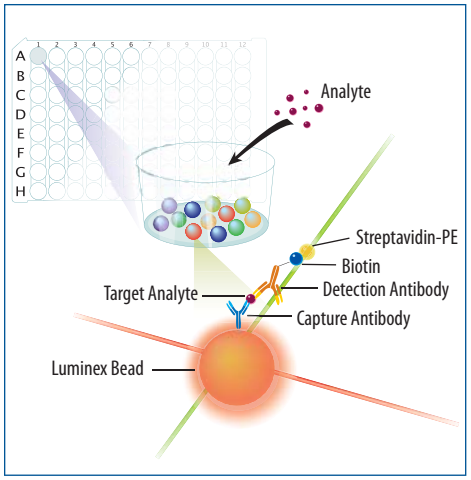


Non-traditional Notch Activation in Colorectal Cancer Cells. TACE/ADAM17 mediates endothelial cell shedding of Jagged 1. Jagged 1 can bind Notch on colorectal cancer cells. This results in the sequential proteolytic cleavage of Notch, culminating in γ-Secretase-mediated release of the Notch intracellular domain (NICD). The NICD interacts with DNA-associated proteins such as CSL to induce the transcription of Notch target genes that promote characteristics of cancer stem cells.

NEW TOOLS: Luminex Screening & Performance Assays – Bead-based Multiplex Kits

Multiplex assays simultaneously detect the levels of multiple proteins in a single sample. As a result, they provide researchers with a cost-effective strategy to obtain more information in less time than single analyte assays. R&D Systems offers several different formats for multianalyte profiling including bead-based Luminex® Screening and Performance Assays. These assays utilize color-coded beads that are coated with analyte-specific antibodies. Beads recognizing different target analytes are mixed prior to the assay and then incubated with the sample. Captured analytes are subsequently detected using a cocktail of biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate. Luminex Screening and Performance Assays come in two formats, polystyrene or magnetic beads. Polystyrene beads are designed for use with the Luminex 100™, Luminex 200™, or Bio-Rad® Bio-Plex® dual laser analyzers. Magnetic beads can be used with any of these instruments or with the Luminex MAGPIX® analyzer.



Luminex Screening Assays are our most flexible kits for multianalyte profiling of cell culture supernates, serum, or plasma samples. Kits include a multi-purpose diluent that allows up to 40 user-defined target analytes to be simultaneously profiled. Analytes are selected from a broad menu that has recently been expanded to include 39 new target analytes. With the addition of these analytes, researchers can now choose from a list of 98 potential analytes for their experiments. This list will be further expanded in the next several months as we are currently developing assays for 20 more target analytes that will be available in July.

FEATURES

- Flexible: Multiple human biomarkers can be simultaneously profiled
- The most cost-effective assay for profiling multiple markers
- Offers a menu that will be rapidly expanding
- All assays can be performed in 3-3.5 hours
- Assays require a small sample volume (<50 µL)
- Reagents are supplied as premixed kits and shipped in 3-5 days from the date of order

COMING SOON!

A New Online Tool to Simplify Luminex Assay Design & Ordering

ANALYTES AVAILABLE IN THE POLYSTYRENE BEAD FORMAT

Select up to 40 human biomarkers from the list below.

Adiponectin/Acrp30	CD14 *new*	EphA2 *new*	IL-1 RI *new*	Leptin/OB	ST2/IL-1 R4
Aggrecan	CD27/TNFRSF7 *new*	Fas/TNFRSF6/CD95 *new*	IL-1 RII *new*	MMP-1	TACI/TNFRSF13B *new*
Angiopoietin-2 *new*	CD30/TNFRSF8 *new*	Fas Ligand *new*	IL-2	MMP-2	Tie-1 *new*
BAFF/BLyS *new*	CD40/TNFRSF5 *new*	FGF basic	IL-4	MMP-3	Tie-2 *new*
BCMA/TNFRSF17 *new*	Chitinase 3-like 1/YKL40	FGF-21	IL-5	MMP-7	Thrombopoietin/Tpo
BMP-2	Comp. Factor D/Adipsin	Galectin-3 *new*	IL-6	MMP-8	TNF-α
BMP-4	Cripto-1	G-CSF	IL-10	MMP-9	TNF RI/TNFRSF1A *new*
BMP-9	C-Reactive Protein/CRP	GDF-15	IL-12 p70	MMP-12 *new*	TNF RII/TNFRSF1B *new*
Cardiac Myoglobin *new*	CXCL1/GROα	GM-CSF	IL-17A	MMP-13	TRAIL R3/TNFRSF10C *new*
Cardiac Troponin I/cTNI *new*	CXCL5/ENA-78	HB-EGF	IL-18 BPa	PCSK9 *new*	VCAM-1/CD106
CCL2/MCP-1	CXCL8/IL-8	HGF	IL-19	RAGE *new*	VEGF
CCL3/MIP-1α	CXCL10/IP-10	ICAM-1/CD54	IL-22 *new*	ROBO4 *new*	VEGF R1/Flt-1 *new*
CCL4/MIP-1β	CXCL11/I-TAC	IFN-γ	IL-23	E-Selectin/CD62E	VEGF R3/Flt-4 *new*
CCL5/RANTES	CXCL13/BLC/BCA-1 *new*	IFN-γ R1/CD119 *new*	IL-27	P-Selectin/CD62P	
CCL8/MCP-2 *new*	DcR3/TNFRSF6B *new*	IL-1α/IL-1F1	IL-28A/IFN-λ2	Serpin E1/PAI-1 *new*	
CCL13/MCP-4 *new*	Dkk-1 *new*	IL-1β/IL-1F2	IL-31 *new*	SHBG *new*	
CCL20/MIP-3α *new*	EGF	IL-1ra/IL-1F3	IL-33	SOST *new*	

ANALYTES AVAILABLE IN THE MAGNETIC BEAD FORMAT

Select up to 25 phospho-RTKs from one of the panels below.

Human Phospho-RTK Kit A

EGF R/ErbB1	EphB1	HGF R/c-MET
EphA1	EphB2	IGF-I R
EphA2	EphB3	M-CSF R
EphA3	EphB4	MSP R/Ron
EphA4	EphB6	Ret
EphA5	ErbB2/Her2	SCF R/c-kit
EphA6	ErbB3/Her3	TrkA
EphA7	ErbB4/Her4	
EphA8	Flt-3/Flk-2	

Human Phospho-RTK Kit B

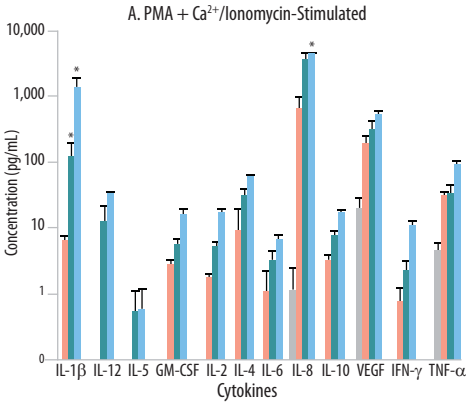
ALK/CD246	Insulin R/CD220	Tie-1
Axl	LTK	Tie-2
DDR1	Mer	TrkB
DDR2	MuSK	TrkC
Dtk	PDGF Rα	VEGF R1/Flt-1
FGF R1	PDGF Rβ	VEGF R2/KDR
FGF R2α	PTK7/CCK4	VEGF R3/Flt-4
FGF R3	ROR1	
FGF R4	ROR2	

Analytes shown in blue are only available from R&D Systems.

Luminex Performance Assays are our most accurate and precise bead-based kits for simultaneously quantifying multiple analytes in qualified complex matrices. These kits utilize panel-optimized diluents that provide maximum performance for up to 22 analytes, depending on the panel selected. Analytes can be ordered either as a complete panel or as a smaller subset from any of the panels shown below. The kits can be used to measure target analytes in qualified sample types, which may include cell culture supernates, serum, plasma, urine, saliva, and milk.

FEATURES

- Kits undergo similar validation testing as the Quantikine® single analyte assays and are correlated with Quantikine ELISAs, when available
- All analytes in a given panel are tested together to ensure optimal kit performance for the entire panel
- Kits consist of base kits and bead sets, which can be ordered individually or as premixed kits
- All assays can be performed in 2.5-5 hours
- Assays require a small sample volume (<100 µL)
- Kits containing beads that the user will mix are delivered 1 day after the order is placed; kits containing beads that we mix are delivered in 2-4 days



Multiplex Measurement of PMA/Ca^{2+}/Ionomycin or LPS-stimulated Cytokine Production in Cultured Whole Blood. Whole blood samples from apparently healthy human donors were cultured for one, eight, or twenty-four hours in the presence of A) phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and Ca^{2+}/Ionomycin (500 ng/mL) or B) lipopolysaccharide (LPS; 50 ng/mL). Samples were taken from unstimulated and stimulated cultures and centrifuged to collect plasma. The levels of the indicated cytokines were simultaneously quantified using the Luminex Performance Human High Sensitivity Cytokine Panel A (Catalog # LHSC000). Columns represent the mean of four whole blood samples + the standard deviation. *One or more of the results were above the range of the standard curve and the value was extrapolated.

ANALYTES AVAILABLE IN THE POLYSTYRENE BEAD FORMAT

Human Biomarker Panel A
BAFF/BLyS
CCL20/MIP-3α
CD14
CD27/TNFRSF7
CXCL13/BLC/BCA-1
gp130
IL-2 Rα
IL-6 Rα
TNF RII/TNFRSF1B

Human Cardiac Panel A
CD40 Ligand/TNFSF5
GDF-15
Pappalysin-1/PAPP-A
PCSK9
ST2/IL-1 R4
TNF RII/TNFRSF1B

Human Cardiac Panel B
C-Reactive Protein/CRP
Cystatin C
Myeloperoxidase/MPO
P-Selectin/CD62P
Serpin E1/PAI-1
TIMP-1

Human Cytokine Panel B
CCL11/Eotaxin
CD40 Ligand/TNFSF5
CXCL10/IP-10
CXCL11/I-TAC
EGF
HGF
IL-12 p70
IL-13
Leptin/OB

Human Obesity Panel
Adiponectin/Acrp30
C-Reactive Protein/CRP
CCL2/MCP-1
Comp. Factor D/Adipsin
IL-6
IL-10
Leptin/OB
Resistin
Serpin E1/PAI-1
TNF-α

Rat Kidney Toxicity Panel
Cystatin C
FABP1/L-FABP
Lipocalin-2/NGAL
Osteopontin
TIM-1/KIM-1/HAVCR

Mouse Cytokine Panel
CCL2/JE/MCP-1
CXCL1/KC
CXCL2/MIP-2
GM-CSF
IFN-γ
IL-1β/IL-1F2
IL-2
IL-4
IL-5
IL-6
IL-10
IL-12 p70
IL-13
IL-17
TNF-α
VEGF

Human Adhesion Molecule 4-plex*
ICAM-1/CD54
E-Selectin/CD62E
P-Selectin/CD62P
VCAM-1/CD106

Rat Cytokine Panel
CXCL2/CINC-3
CXCL3/CINC-2α/β
GM-CSF
ICAM-1/CD54
IFN-γ
IL-1α/IL-1F1
IL-1β/IL-1F2
IL-2
IL-4
IL-6
IL-10
IL-13
IL-18/IL-1F4
L-Selectin/CD62L
TIMP-1
TNF-α
VEGF

Multi-species TGF-β 3-plex*
TGF-β1
TGF-β2
TGF-β3

ANALYTES AVAILABLE IN THE MAGNETIC AND POLYSTYRENE BEAD FORMAT

Human Angiogenesis Panel
Angiogenin
Angiopoietin-1
Endostatin
FGF acidic
FGF basic
PDGF-AA
PDGF-BB
PIGF
Thrombospondin-2
VEGF
VEGF-D

Human Cytokine Panel A
CCL2/MCP-1
CCL3/MIP-1α
CCL4/MIP-1β
CCL5/RANTES
CXCL5/ENA-78
CXCL8/IL-8
FGF basic
G-CSF
GM-CSF
IFN-γ
IL-1α/IL-1F1
IL-1β/IL-1F2
IL-1ra/IL-1F3
IL-2
IL-4
IL-5
IL-6
IL-10
IL-17
TNF-α
Thrombopoietin/Tpo
VEGF

Human High Sensitivity Cytokine Panel A
CXCL8/IL-8
GM-CSF
IFN-γ
IL-1β/IL-1F2
IL-2
IL-4
IL-5
IL-6
IL-10
IL-12 p70
TNF-α
VEGF

Human MMP Panel
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-10
MMP-12
MMP-13
EMMPRIN

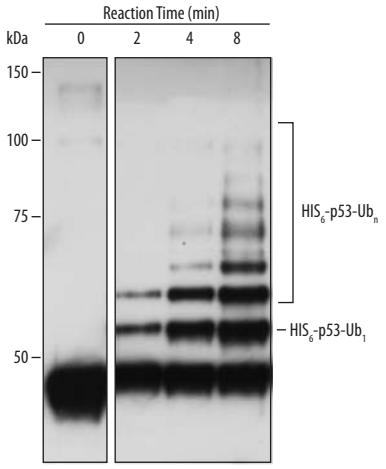
Human TIMP 4-plex*
TIMP-1
TIMP-2
TIMP-3
TIMP-4

*These kits are only available as premixed kits.

TECHNICAL NOTE: *In Vitro* MDM2/p53 Ubiquitination Kit (Catalog # K-200B)

The tumor suppressor protein p53 is a transcription factor that promotes cell cycle arrest, senescence and apoptosis, and it is one of the most commonly mutated genes in human cancers. The abundance of p53 protein in cells is regulated by MDM2, a Ubiquitin ligase (E3) that directly ubiquitinates p53 and promotes its degradation.

The MDM2/p53 Ubiquitination Kit reproducibly and reliably generates MDM2-ubiquitinated p53 that can be used for many downstream applications. It is ideal for use as a Western blot control or to investigate enzymes that act upon ubiquitinated p53. For examples of ubiquitinated p53 used in this manner please see: Wu, H. *et al.* (2011) Nat. Med. **17**:347 and Faesen, A. *et al.* (2011) Med. Cell **44**:147.



***In Vitro* Ubiquitination of p53 by MDM2.** An *in vitro* ubiquitination reaction containing the GST-MDM2, His_6-p53, E1 and E2 enzymes, Ubiquitin, reaction buffer, and Mg^{2+}-ATP provided in the Human MDM2/p53 Ubiquitination Kit (Catalog # K-200B) was carried out for the indicated times. Ubiquitinated p53 was detected by Western blot using the monoclonal p53-specific antibody included in the kit. The ladder of slower migrating bands represents ubiquitinated p53. The '0' timepoint was obtained from a separate reaction containing all components except Mg^{2+}-ATP.

KIT FEATURES

- Includes all reagents required to perform the *in vitro* ubiquitination reaction
- Provides a positive control for p53 ubiquitination experiments
- Generates ubiquitinated p53 that can be used for downstream experiments

UBIQUITINATION KITS	CATALOG #	UBIQUITINATION KITS	CATALOG #
CHIP/Luciferase	K-280	MDM2/p53	K-200B
E6AP/S5a	K-230	MuRF1/S5a	K-102
E6AP/E6/p53	K-240	Parkin/Auto-ubiquitination	K-105
MDM2/S5a	K-210	RNF4/di-SUMO3	K-220

To learn more about ubiquitination & view up-to-date product listings, please visit www.BostonBiochem.com

Learn more about the ubiquitination of your substrate with Ubiquitin mutants

Ubiquitin mutants are ideal to use as negative controls (Figure 1), distinguish multi-mono-ubiquitination from poly-ubiquitination (Figure 2), and determine the linkage of poly-Ubiquitin chains (Figure 3). To view all available recombinant human Ubiquitin mutants, please visit www.BostonBiochem.com.

FIGURE 1

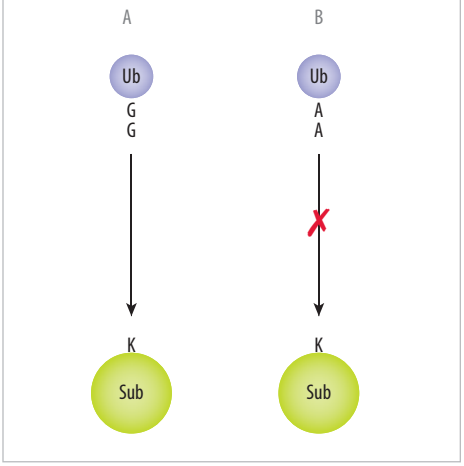


Figure 1. Inactive C-terminal Ubiquitin Mutants are an Ideal Negative Control for Substrate Ubiquitination. Ubiquitin is covalently attached to substrate proteins via its C-terminal glycine residue (A). Inactive C-terminal Ubiquitin mutants, such as the Ubiquitin-AA Mutant (Catalog # UM-HAA) that has both C-terminal glycine residues mutated to alanine, cannot be attached to substrate proteins (B).

FIGURE 2

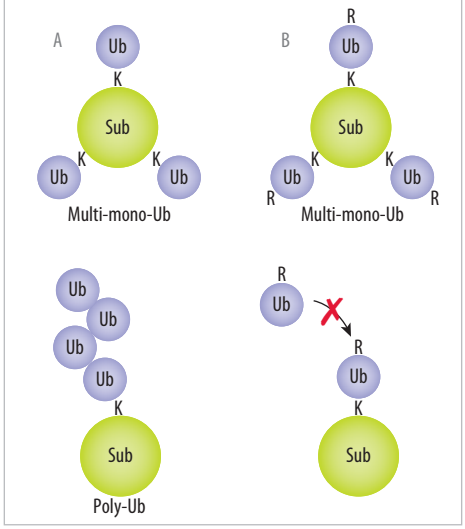


Figure 2. Distinguish Between Multi-Mono-Ubiquitination and Poly-Ubiquitination with the No K Ubiquitin Mutant. Wild-type Ubiquitin can generate both multi-mono-ubiquitinated (A; top) and poly-ubiquitinated substrates (A; bottom). In contrast, the No K Ubiquitin Mutant (Catalog # UM-NOK), which has all seven of its lysines mutated to arginine, can generate multi-mono-ubiquitinated substrates (B; top), but not poly-ubiquitinated substrates (B; bottom).

FIGURE 3

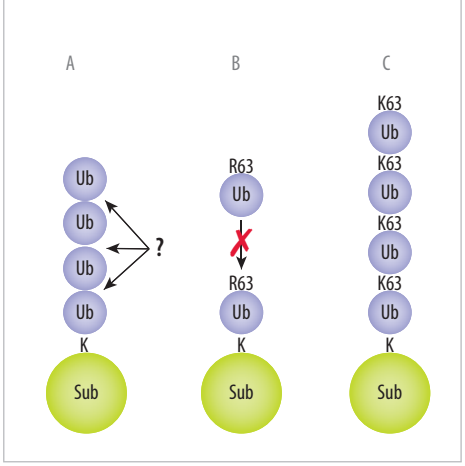


Figure 3. Determine Ubiquitin Chain Linkage with Lysine to Arginine and Single Lysine Ubiquitin Mutants. Ubiquitin chain linkage cannot be determined using wild-type Ubiquitin (A). Lysine to arginine mutants, such as Ubiquitin Mutant K63R (Catalog # UM-K63R), block the formation of poly-Ubiquitin chains that utilize the mutated lysine (B). Single lysine mutants, such as Ubiquitin Mutant with K63 Only (Catalog # K630), can be used to verify that poly-Ubiquitin chains are linked via a particular lysine (C).

Continued from page 1

In order to determine the functional significance of Akt-dependent Beclin 1 phosphorylation, a mutant Beclin 1 with both Akt phosphorylation sites converted to alanine was constructed (Beclin 1 AA).[6] Expression of Beclin 1 AA prevented myr-Akt-dependent autophagy suppression in Rat2 fibroblast cells. In addition, Beclin 1 AA expression partially inhibited myr-Akt-dependent transformation of Rat2 cells as determined by an anchorage-independent growth assay. The tumor growth rate and volume in mice injected with myr-Akt-expressing Rat2 cells was also reduced by expression of Beclin 1 AA. Collectively, these results suggest that Akt-dependent phosphorylation of Beclin 1 suppresses autophagy and may promote cellular transformation and tumor growth.

Beclin 1 and Beclin 1 AA were then immunoprecipitated from HeLa cells to identify proteins that only interact with Beclin 1 following phosphorylation by Akt. 14-3-3 proteins, which are known to bind phospho-Ser/Thr, co-immunoprecipitated with Beclin 1, but not Beclin 1 AA.[6,10] Consistent with previous results showing that 14-3-3 proteins bind to cytoskeletal components, Beclin 1 also pulled down the intermediate filament proteins, Vimentin and K18.[11] The significance of Vimentin in this pathway was investigated via siRNA knockdown experiments. Depletion of Vimentin in Rat2 cells suppressed autophagy and promoted cellular transformation, similar to the results obtained from Beclin 1 AA expression. This suggests that Akt-dependent Beclin 1 phosphorylation and the subsequent interaction of phosphorylated Beclin 1 with Vimentin are both part of the same pathway.

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The data presented by Wang *et al.* are consistent with a model in which Akt-phosphorylated Beclin 1 is sequestered by intermediate filaments via its interaction with 14-3-3 proteins, resulting in autophagy suppression and tumor growth. In support of this model, autophagy has been reported to have anti-tumor activities in certain contexts.[12] These results also suggest that blocking the interaction between Akt and Beclin 1 could enhance the efficacy of cancer therapies that activate autophagy.

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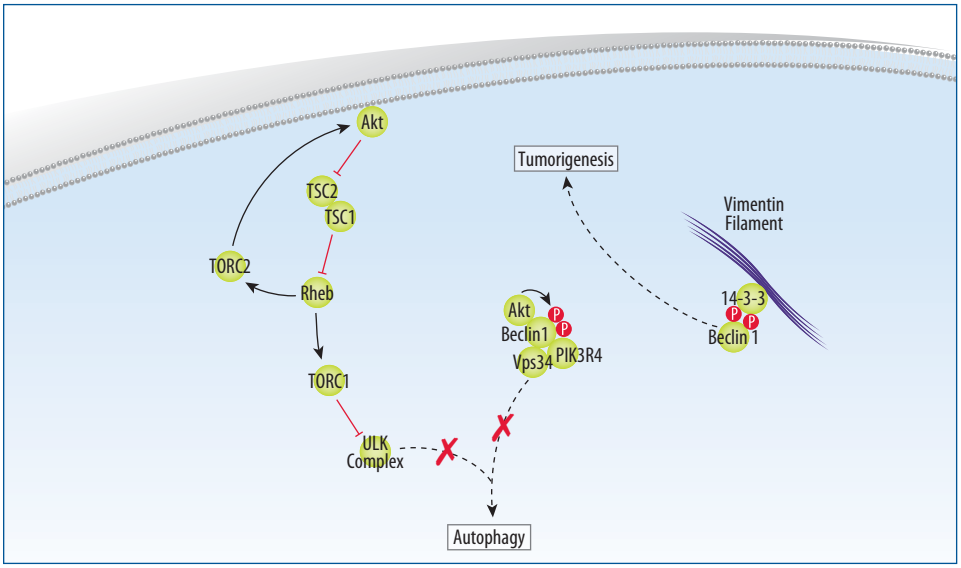

Akt Directly Phosphorylates Beclin 1 to Suppress Autophagy. Autophagy initiation is regulated by the Unc-51-like Kinase (ULK) kinase complex and a complex of core autophagy proteins that includes Beclin 1, Vps34, and PIK3R4. Under growth promoting conditions, or if Akt is constitutively active, autophagy can be inhibited in an Akt-dependent manner via TORC1 activation. Akt also directly phosphorylates Beclin 1 on Ser234 and Ser295, which creates a phospho-binding site for 14-3-3 proteins. The Beclin 1/14-3-3 complex interacts with Vimentin filaments, which might sequester Beclin 1 from other core autophagy proteins. Regulation of Beclin 1 by Akt suppresses autophagy and partially mediates the oncogenic capability of a constitutively active form of Akt.[6]

Continued from page 3

tumor recurrence and represent major roadblocks to conventional cancer therapy. The CSC hypothesis continues to evolve, and understanding the various mechanisms that control the context-dependent regulation of these cells will be important for the generation of targeted, multifaceted approaches to cancer treatment.

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Sample: A431 epithelial carcinoma cell lysates

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