UNIVERSITY OF CALIFORNIA, SAN DIEGO

BioID Utilized to Identify Proteins Mediating Signaling Crosstalk between IKKbeta and STAT3

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in

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by

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The Thesis of Juyeon Ko is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

CHIP	Carboxy Terminus Of Hsp70-Interacting Protein
E	Glutamic acid
HECT	Homologous to E6-associated protein C Terminus
HERC2	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase
HIF1 α	Hypoxia-Inducible Factor 1 α
HRP	Horseradish Peroxidase
HUWE1	HECT, UBA and WWE Domain Containing 1
IGF2BP1	Insulin like growth factor 2 mRNA binding protein 1
IKK	Inhibitor of κB kinase
Interleukin-6	IL-6
IκB	Inhibitor of κB
JAK	Janus kinase
K	Lysine
LRPPRC	Leucine rich pentatricopeptide repeat containing
MM	Multiple Myeloma
MS	Mass Spectrometry
NEMO	NF-κB essential modulator
NF-κB	Nuclear Transcription factor-kappaB
R	Arginine
RING	Really interesting new gene
STAT3	Signal Transducer and Activator of Transcription 3

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ABSTRACT OF THE THESIS

BioID Utilized to Identify Proteins Mediating Signaling Crosstalk between IKKbeta and STAT3

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Professor Daniel J. Donoghue, Chair

IKKβ, which is the main protein kinase in the canonical NF-κB signaling, regulates cytokine production, inflammation, cell proliferation, survival and the responses to cellular stress. In the previous research by Gallo LH. et al. [1] showed that IKKβ K171E mutant undergoes the K-63 linked ubiquitination at K147 site as well as it activates STAT3 signaling pathway. We aimed to investigate the mechanism why which

the mutant IKKβ is mediating the signaling crosstalk, by investigating interacting proteins, specifically an E3 ubiquitin ligase that catalyzing the K-63linked ubiquitination of the mutant IKKβ. We utilized a new technique called BioID that enables the selective capture of proximal interacting proteins by the mutant biotin ligase fused to a protein of interest, the IKKβ K171E 4KR. Here, the results of the identified interacting proteins including E3 ubiquitin ligases which may be involved in the signaling crosstalk are shown. This project has potential to characterize the mechanism by identifying the E3 ligase, which is a promising cancer therapeutics for patients expressing activated mutants of IKKβ which were originally identified in the hematopoietic cancers patients.

INTRODUCTION

The NF-κB pathway

The transcription factor nuclear factor-kappaB (NF-κB) plays critical roles in responses to cellular stress, proliferation, cell survival, cytokine production and inflammation [2]. Physiologic activation of NF-κB occurs when antigens or cytokines such as tumor necrosis factor and interleukin 1β bind to immune receptors. Inhibitor of κB (IκB) kinase (IKK) complex, which consists of catalytic kinase subunits, IKKα and IKKβ, and NEMO (NF-κB essential modulator also known as IKKγ), becomes activated by the upstream kinase, TGF-β activated kinase-1 [3]. Activated IKKβ phosphorylates IκB, which induces degradation of IκB and releases NF-κB subunits to translocate into the nucleus and induce target gene expression [2]. The NF-κB pathway has been reported to be involved in many inflammatory diseases and cancers including leukemia, lymphoma, breast, colon and ovarian cancer [4].

Dysregulation of IKKβ linked to STAT3 pathway

Previous project by Gallo LH. et al. [1] has described that somatic mutations of Lys (K) 171 site altered to either Glu (E) or Arg (R) exhibit constitutive kinase activity of IKKβ, which were initially identified in patient sample of Multiple Myeloma (MM), and Splenic Marginal Zone Lymphoma, and Mantle Cell Lymphoma.

The study also showed that the K171E IKKβ mutant results in increased K63-linked ubiquitination as well as constitutive activation of Signal Transducer and Activator of Transcription 3 (STAT3) pathway without exogenous addition of Interleukin-6 (IL-6)

ligand.

STAT3 pathway is one of the main signaling pathway that's frequently involved in many cancers. Environmental factors such as ultraviolet radiation, carcinogens, infection, stress and nicotine activates receptor and non-receptor tyrosine kinases. Growth factors and cytokines such as IL-6 activate associated Janus kinase (JAK), which in turn phosphorylate STAT3. The activated STAT3s dimerize and translocate to the nucleus to induce target gene transcription and regulate cell proliferation, survival and other different functions [5]. Persistent activation of the receptor- and non-receptor- kinases have shown to up-regulate STAT3, produce more cytokines that activates STAT3, forming a positive tumorigenic feedback loop [6].

Studies have shown that aberrant regulation of STAT3 signaling is implicated in diverse types of cancer, including MM [7], lymphoma, gastric cancer [8], Non-small cell lung cancer [5], and breast cancer [9].

Signaling Crosstalk between the NF-κB and STAT3

Many studies have shown that NF-κB and STAT3 cooperatively regulate a number of cellular responses including immune response, cell survival, proliferation as well as chemo-resistance [10]. Studies have shown that down-regulation of NF-κB and STAT3 activation results in decreased oncogenic phenotype, shown by the decreased MM cell adhesion to bone marrow stromal cells, and cytokine secretion that constitutively activating NF-κB and STAT3 in MM cells [10] and by the reduced Cluster of differentiation 44-positive cell population, one of the cancer stem cell markers, in breast cancer cell lines [11].

Importance of Ubiquitination

Ubiquitination is one of the post-translational modifications occur to widen the range of functions of the proteins. Ubiquitin is a highly conserved 76-amino acid polypeptide expressed in all cells and it has seven lysine residues that can be polymerized into various ubiquitin linkages. Ubiquitination is a multistep process that begins with the activation of ubiquitin with adenosine triphosphate by an E1 ubiquitin activating enzyme. It is followed by the formation of a thioester linkage between the transferred ubiquitin and the cysteine in the active site of an E2 ubiquitin conjugating enzyme. An E3 ubiquitin ligase then catalyzes the transfer of the ubiquitin from E2 to the lysine site of a specific target substrate.

There are three major types of E3 ligases: Really Interesting New Gene (the RING finger) E3, the U-box E3, and Homologous to E6-associated protein C Terminus (HECT) domain E3. While RING type and U-Box type E3 ligases simply facilitate the direct transfer of ubiquitin from E2 to a substrate, HECT type E3 ligases transfer the ubiquitin from E2 to its catalytic cysteine residue and then catalyze the transfer to a target substrate. [12].

Depending on the type of the linkage, Ubiquitination results in different functions. For example, K48-linked Ub chains result in proteasomal degradation. On the other hand, K63-linked Ubiquitination serves as a stabilizing signal and scaffolding interactions. [13] There are about 600 E3 ligases that are encoded by the human genome. And so far, not many E3 ligases have been shown to target substrates for K63-linked ubiquitination, but, among them are TRAF6 for immune response [14], HUWE1[14] and CHIP [15] for stabilizing targets, Nedd4 [16] and β-TRCP [13] for endocytosis, RNF4 [17], RNF8 [14]

and RNF168 [18] for DNA repair response.

In the previous report by Gallo LH. et al. [1] showed that IKKβ K171E mutant undergoes the K-63 linked ubiquitination at K147 site. Here, results of the process of identifying the E3 ubiquitin ligase that catalyze this signaling are shown.

RESULTS

Mutant IKKβ constructs by Quikchange site-directed mutagenesis

Based on the previous mass spectrometry (MS) data, we generated IKKβ 4KR (K301R, K418R, K555R and K703R) mutant in WT and K171E background by Quikchange site-directed mutagenesis. The mutant constructs were named IKKβ WT 4KR and IKKβ K171E 4KR, respectively (Figure 1). The importance of K147 site for the kinase activity and K-63 linked ubiquitination was examined in both IKKβ WT 4KR and K171E 4KR by immunoblot (data not shown). Although four Lys sites were altered to Arg, the differences between K147 intact 4KR construct and the K147R 4KR were consistent in both the kinase activity and the K-63 linked ubiquitination.

BioID method with MS analysis identifies interacting proteins of the mutant IKKβ

We aimed to identity which E3 ligase is poly-ubiquitinating this IKKβ K171E 4KR mutant at K147 site, and if any other sets of proteins are recruited by this scaffolding signal, by utilizing the BioID method, named for proximity-dependent biotin identification, which was developed in 2012 by Roux group [19]. This technique utilizes a mutant biotin ligase (BirA R118G, BirA*) fused to a protein of interest that labels interacting endogenous proteins in proximity in cells. Then the biotinylated proteins can be selectively isolated because the biotinylation is rare protein modification in nature. And they can be purified with standard biotin-affinity capture. This method has advantages in that it can be applied to insoluble proteins, and it can also identify weak and/or transient interactions. By using the K171E 4KR mutants, two independent cell

lines that stably expressing myc-BioID-IKKβ K171E 4KR and IKKβ K171E 4KR-BioID-HA were generated (Figure 2). Untransfected cells to which biotin was not added were subjected to the same experiment condition and analysis was performed to compare for the endogenously biotinylated proteins. The result showed a significant difference between two cell lines in that N-terminus BioID fusion construct gave more robust data, suggesting that N-terminus region of IKKβ interacts with more and diverse proteins than the C-terminus region (Figure 3).

The biotinylated proteins were analyzed by MS to reveal the identity of 2469 proteins interacting with IKKβ K171E 4KR. The proteins were sorted by their abundance represented by the normalized spectral count (Table 1). We selected several proteins to further look into based on their known functions that associated with the signaling pathways in cancers as well as their abundance. Leucine rich pentatricopeptide repeat containing (LRPPRC), Insulin like growth factor 2 mRNA binding protein 2 (IGF2BP1), and HECT, UBA and WWE Domain Containing 1 (HUWE 1) E3 ubiquitin protein ligase were selected to investigate the importance in signaling crosstalk between IKKβ mutant and STAT3.

Validation of the candidates

To begin to characterize the new putative recruited proteins and E3 ubiquitin protein ligase for the K-63 linkage scaffolding signal, we utilized siRNA method. Cells were transfected with siRNA of LRPPRC, IGF2BP1 and HUWE1, respectively, and also transiently transfected with the mutant IKKβ K171E 4KR and finally lysed and analyzed by Western Blot. In the experiments, non-targeting control siRNA was used as a negative

control and the control siRNA did not show effect on each protein of interest. First, siRNA-mediated *LRPPRC* knockdown, in the presence of the mutant IKKβ, it was shown to slightly up-regulate the activation of STAT3. This suggests LRPPRC may have a role inhibiting the constitutive activation of the STAT3 (Figure 4). Second, siRNA-mediated inhibition of *IGF2BP1* had no apparent effect on the level of the activation of STAT3 by the mutant IKKβ (Figure 5). Last, we hypothesized that knockdown of *HUWE1* would ablates the STAT3 activation. However, there was no detectable decrease in the constitutive activation of STAT3 by the mutant IKKβ. This showed that it is not the HUWE1 ligase that is catalyzing the K-63linked ubiquitination signal. The effect of the HUWE1 deficiency was examed for any alterations in STAT3 with endogenous wildtype IKKβ in the presence or the absence of IL-6, to show no apparent effect as well (Figure 6).

Other candidate E3 ligases

From the same BioID sample of N-terminus fusion BioID IKKβ K171E 4KR, we obtained a list of 272 phosphorylated peptides sorted in the order of intensity which also represents protein abundance. Although HUWE1 was the most abundant E3 ligase which interacts the same N-terminus fusion BioID IKKβ K171E 4KR in the total peptide list, the phosphorylated peptide data suggest that Carboxy Terminus of Hsp70-Interacting Protein (CHIP also known as STUB1) and HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase 2 (HERC2) to be more abundant in the phosphorylated state (Table 2).

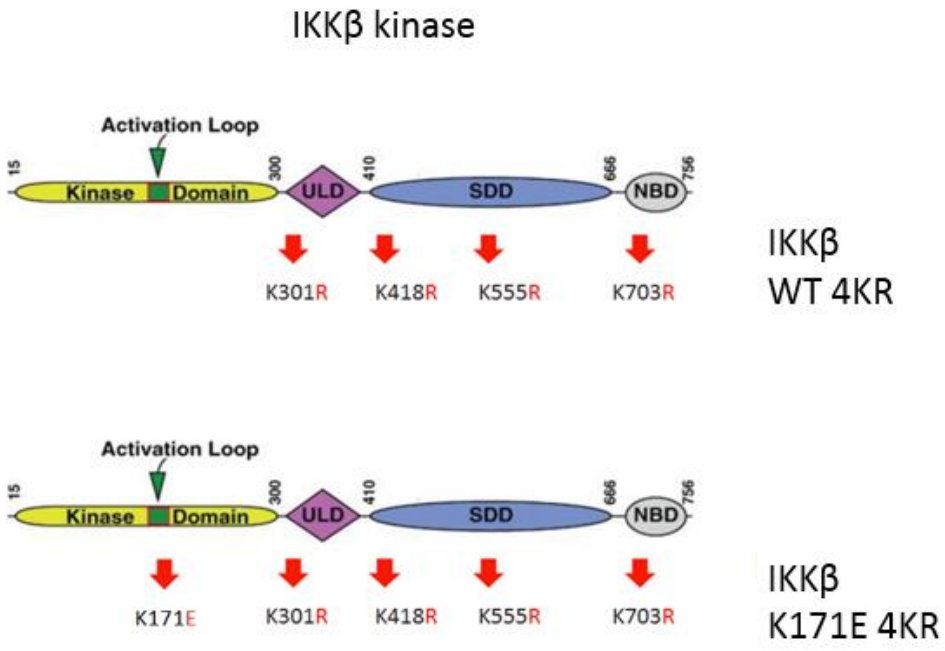

Figure 1. Schematic of IKKbeta WT 4KR and K171E 4KR constructs.

The top one represents 4 Lys sites (K301, 418, K555, and K703) are mutated to Arg. The bottom one shows that same 4 Lys sites were mutated to Arg as wells as the Lys 171 site is mutated to Glu.

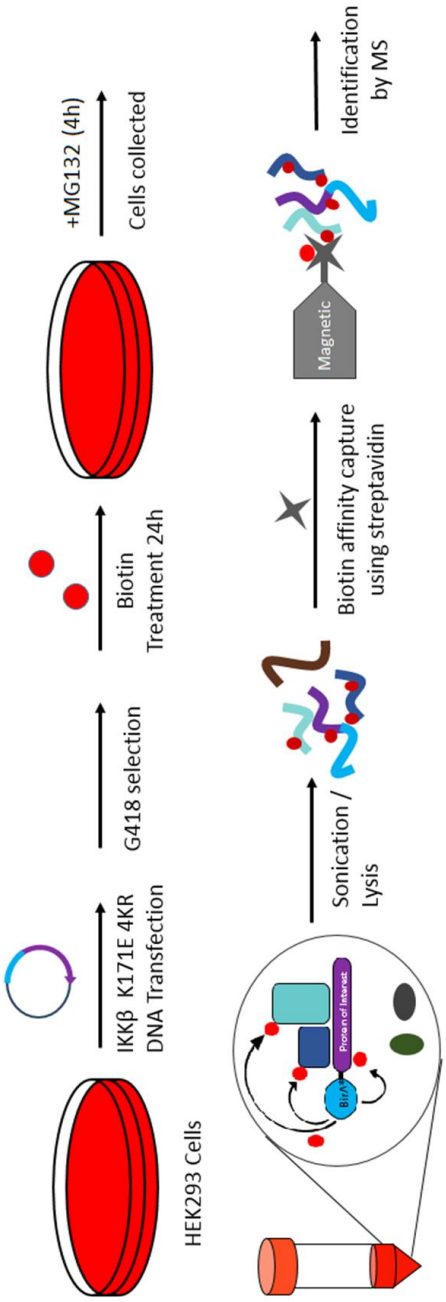

Figure 2. BioID work flow using the N-terminus fusion BioID IKKβ K171E 4KR.

By using the K171E 4KR mutants, two independent HEK293 cell lines that stably expressing myc-BioID-IKKβ K171E 4KR and IKKβ K171E 4KR-BioID-HA were generated. After adding biotin (~20h prior to the lysis) and MG132 (~4h prior to the lysis), cells were collected and lysed by sonication and biotinylated proteins were captured onto streptavidin conjugated magnetic beads. The beads were collected onto a magnetic stands and the captured proteins were subjected to the MS analysis.

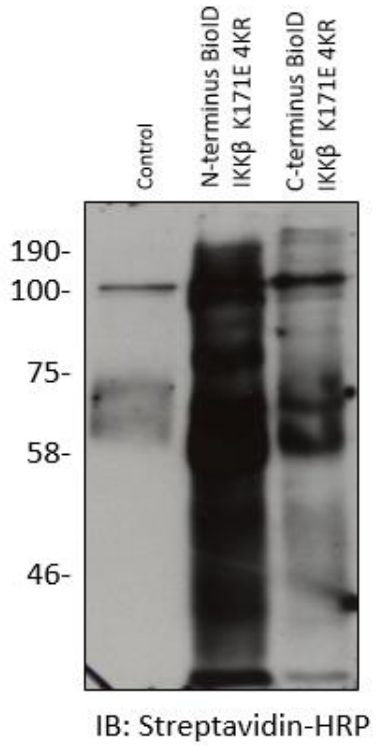

Figure 3. Immunoblot of biotinylated proteins by fusion IKKβ.

Lysates from the BioID cell lines were immunoblotted using Streptavidin Horseradish Peroxidase (HRP) Conjugate. Streptavidin binds specifically with biotin to detect the biotinylated proteins in the lysates. The control showed endogenously biotinylated proteins. The N-terminus fusion BioID IKKβ K171E 4KR showed more robust signal than the C-terminus fusion BioID IKKβ K171E 4KR.

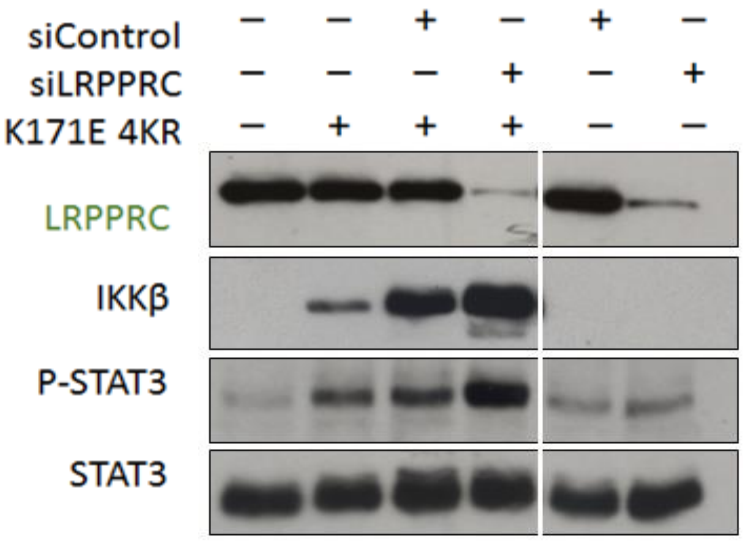

Figure 4. Immunoblot of siRNA-mediated knockdown of LRPPRC analysis.

Lysates were examined for the LRPPRC expression (top panel), IKKβ K171E 4KR (second panel), and phospho-STAT3 (third panel), and STAT3 (bottom panel). The membrane was stripped and reprobed for all.

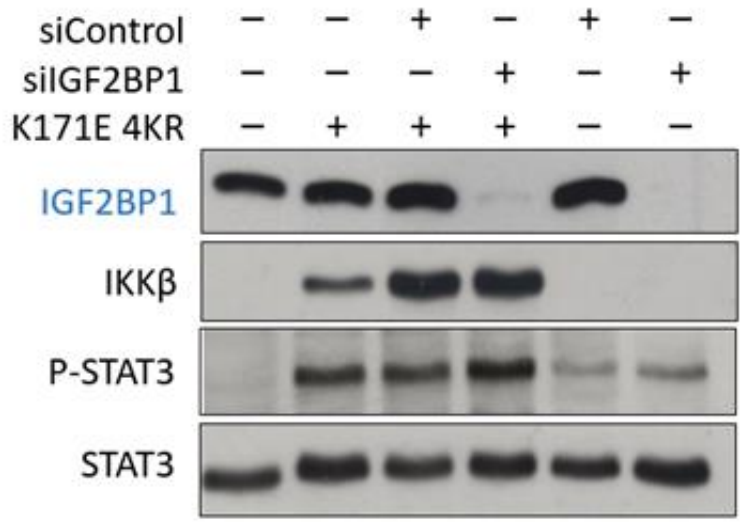

Figure 5. Immunoblot of siRNA-mediated knockdown of IGF2BP1 analysis.

Lysates were examined for the IGF2BP1 expression (top panel), IKKβ K171E 4KR (second panel), and phospho-STAT3 (third panel), and STAT3 (bottom panel). The membrane was stripped and reprobed for all except the top panel.

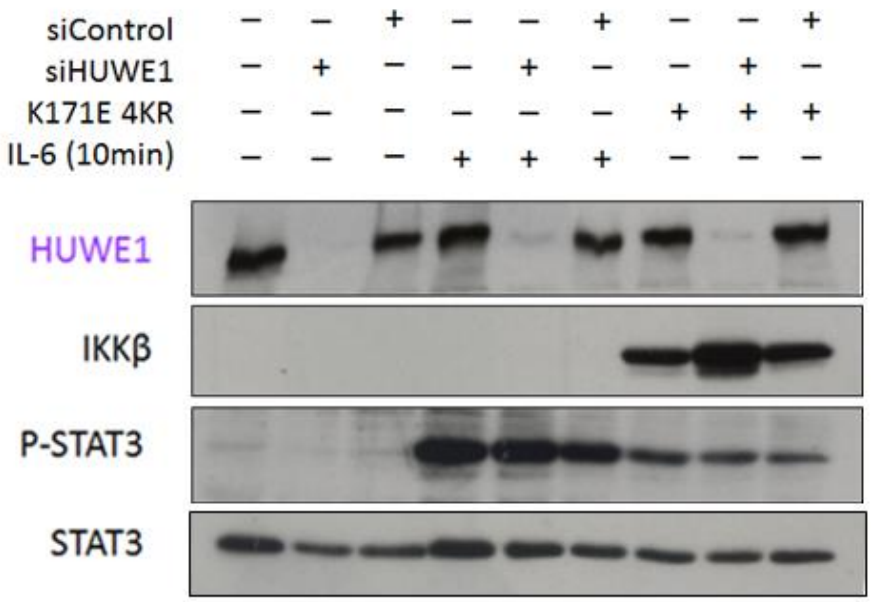

Figure 6. Immunoblot of siRNA-mediated knockdown of HUWE1 analysis.

Lysates were examined for the HUWE1 expression (top panel), IKKβ K171E 4KR (second panel), and phospho-STAT3 (third panel), and STAT3 (bottom panel). The membrane was stripped and reprobed for all.

Table 1. Interacting proteins identified by MS.

Top 100 proteins sorted by the normalized spectral count are shown here.

	IPI#	Proteins Identified	Spectral Count	Normalized Spectral Count
1	IPI00024708B	JK1 Myctag BirA IKKbeta K171E 4KR K301R K418R K555R K703R	2659	0.023414935
2	IPI00024708C	JK2 IKKbeta K171E 4KR K301R K418R K555R K703R BirA HA TAG	2633	0.023185981
3	IPI00744115	PCCA propionyl-CoA carboxylase alpha chain, mitochondrial isoform a precursor	2032	0.017893625
4	IPI00296337	PRKDC Isoform 2 of DNA-dependent protein kinase catalytic subunit	1696	0.014934836
5	IPI00299402	PC Pyruvate carboxylase, mitochondrial	1553	0.01367559
6	IPI00304925	HSPA1B;HSPA1A cDNA FLJ54392, highly similar to Heat shock 70 kDa protein 1	1314	0.011570976
7	IPI00645452	TUBB Tubulin, beta	1291	0.01136844
8	IPI00456969	DYNC1H1 Cytoplasmic dynein 1 heavy chain 1	1127	0.009924269
9	IPI00302592	FLNA Isoform 1 of Filamin-A	1126	0.009915463
10	IPI00024580	MCCC1 Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	1054	0.009281437
11	IPI00911039	HSPA1B;HSPA1A cDNA FLJ54408, highly similar to Heat shock 70 kDa protein 1	983	0.008656217
12	IPI00026781	FASN Fatty acid synthase	980	0.008629799
13	IPI00844578	DHX9 ATP-dependent RNA helicase A	944	0.008312786
14	IPI00013475	TUBB2A Tubulin beta-2A chain	930	0.008189503
15	IPI00218343	TUBA1C Tubulin alpha-1C chain	864	0.007608313
16	IPI00015953	DDX21 Isoform 1 of Nucleolar RNA helicase 2	828	0.0072913
17	IPI00930130	TUBB6 cDNA FLJ11352 fis, clone HEMBA1000020, highly similar to Tubulin beta-2C chain	786	0.006921451
18	IPI00186290	EEF2 Elongation factor 2	777	0.006842198
19	IPI00024067	CLTC Isoform 2 of Clathrin heavy chain 1	764	0.006727721
20	IPI00410402	TUBA3E Tubulin alpha-3E chain	700	0.006164142
21	IPI00007928	PRPF8 Pre-mRNA-processing-splicing factor 8	577	0.005081014
22	IPI00643152	HSPA1L Heat shock 70 kDa protein 1-like	560	0.004931314
23	IPI00017617	DDX5 Probable ATP-dependent RNA helicase DDX5	541	0.004764001
24	IPI00019502	MYH9 Isoform 1 of Myosin-9	529	0.00465833
25	IPI00396485	EEF1A1P5;EEF1A1 Putative elongation factor 1-alpha-like 3	524	0.004614301
26	IPI00941328	HSPA1B;HSPA1A Putative uncharacterized protein HSPA1B	519	0.004570271
27	IPI00465248	ENO1 Isoform alpha-enolase of Alpha-enolase	506	0.004455794
28	IPI00414676	HSP90AB1 Heat shock protein HSP 90-beta	487	0.004288482
29	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	460	0.004050722
30	IPI00449049	PARP1 cDNA FLJ53442, highly similar to Poly (ADP-ribose) polymerase 1	460	0.004050722
31	IPI00218753	TOP2A DNA topoisomerase 2	453	0.003989081
32	IPI00479186	PKM2 Isoform M2 of Pyruvate kinase isozymes M1\M2	448	0.003945051
33	IPI00397526	MYH10 Isoform 3 of Myosin-10	424	0.003733709
34	IPI00290566	TCP1 T-complex protein 1 subunit alpha	419	0.003689679

Table 1. Interacting proteins identified by MS, Continued.

35	IPI00019359	KRT9 Keratin, type I cytoskeletal 9	415	0.003654456
36	IPI00021290	ACLY ATP-citrate synthase isoform 2	405	0.003566397
37	IPI00644079	HNRNPU Isoform Long of Heterogeneous nuclear ribonucleoprotein U	403	0.003548785
38	IPI00015838	LYAR Cell growth-regulating nucleolar protein	402	0.003539979
39	IPI00025491	EIF4A1 Eukaryotic initiation factor 4A-I	399	0.003513561
40	IPI00420014	SNRNP200 Isoform 1 of U5 small nuclear ribonucleoprotein 200 kDa helicase	396	0.003487143
41	IPI00644712	XRCC6 X-ray repair complementing defective repair in Chinese hamster cells 6	395	0.003478337
42	IPI00013881	HNRNPH1 cDNA FLJ54533, highly similar to Heterogeneous nuclear ribonucleoprotein H	394	0.003469532
43	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	387	0.00340789
44	IPI00014898	PLEC Isoform 8 of Plectin-1	386	0.003399084
45	IPI00220834	XRCC5 X-ray repair cross-complementing protein 5	381	0.003355055
46	IPI00167941	MDN1 Midasin	369	0.003249384
47	IPI00003865	HSPA8 Isoform 2 of Heat shock cognate 71 kDa protein	364	0.003205354
48	IPI00396378	HNRNPA2B1 Putative uncharacterized protein HNRNPA2B1	364	0.003205354
49	IPI00027834	HNRNPL Heterogeneous nuclear ribonucleoprotein L	359	0.003161324
50	IPI00221325	RANBP2 E3 SUMO-protein ligase RanBP2	356	0.003134907
51	IPI00848226	GNB2L1 Guanine nucleotide-binding protein subunit beta-2-like 1	355	0.003126101
52	IPI00003886	GNL3;SNORD19B Isoform 1 of Guanine nucleotide-binding protein-like 3	343	0.00302043
53	IPI00889541	DDX17 probable ATP-dependent RNA helicase DDX17 isoform 3	342	0.003011624
54	IPI00382470	HSP90AA1 Isoform 1 of Heat shock protein HSP 90-alpha	341	0.003002818
55	IPI00027107	TUFM Tu translation elongation factor, mitochondrial precursor	340	0.002994012
56	IPI00021812	AHNAK Neuroblast differentiation-associated protein AHNAK	335	0.002949982
57	IPI00216587	SNORD38B;RPS8;SNORD55 40S ribosomal protein S8	333	0.002932371
58	IPI00301263	CAD Putative uncharacterized protein CAD	328	0.002888341
59	IPI00031691	RPL9 Protein	326	0.002870729
60	IPI00908469	TUBB6 cDNA FLJ52712, highly similar to Tubulin beta-6 chain	325	0.002861923
61	IPI00783271	LRPPRC Leucine-rich PPR motif-containing protein, mitochondrial	316	0.00278267
62	IPI00217507	NEFM NEFM protein	315	0.002773864
63	IPI00030275	TRAP1 57 kDa protein	313	0.002756252
64	IPI00215965	HNRNPA1 cDNA FLJ51586, moderately similar to Heterogeneous nuclear ribonucleoprotein A1	310	0.002729834
65	IPI00419373	HNRNPA3 37 kDa protein	306	0.002694611
66	IPI00024279	HEATR1 HEAT repeat containing 1	300	0.002641775
67	IPI00299904	MCM7 Isoform 1 of DNA replication licensing factor MCM7	297	0.002615358
68	IPI00011569	ACACA 268 kDa protein	296	0.002606552
69	IPI00140420	SND1 cDNA FLJ54574, highly similar to Staphylococcal nuclease domain-containing protein 1	293	0.002580134
70	IPI00437751	ACE Isoform Somatic-2 of Angiotensin-converting enzyme	291	0.002562522
71	IPI00400922	PDCD11 Protein RRP5 homolog	291	0.002562522

Table 1. Interacting proteins identified by MS, Continued.

72	IPI00001159	GCN1L1 Translational activator GCN1	290	0.002553716
73	IPI00784090	CCT8 T-complex protein 1 subunit theta	287	0.002527298
74	IPI00025273	GART Isoform Long of Trifunctional purine biosynthetic protein adenosine-3	286	0.002518492
75	IPI00302927	ILK-2;CCT4 T-complex protein 1 subunit delta	284	0.002500881
76	IPI00018465	CCT7 T-complex protein 1 subunit eta isoform d	281	0.002474463
77	IPI00215637	DDX3X ATP-dependent RNA helicase DDX3X	277	0.002439239
78	IPI00910438	SND1 cDNA FLJ54574, highly similar to Staphylococcal nuclease domain-containing protein 1	273	0.002404015
79	IPI00440493	ATP5A1 ATP synthase subunit alpha, mitochondrial	271	0.002386404
80	IPI00006196	NUMA1 NUMA1 variant protein (Fragment)	263	0.002315956
81	IPI00396435	DHX15 Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	259	0.002280733
82	IPI00179964	PTBP1 Isoform 2 of Polypyrimidine tract-binding protein 1	259	0.002280733
83	IPI00026625	NUP155 cDNA FLJ11241 fis, clone PLACE1008603, highly similar to Nuclear pore complex protein Nup155	255	0.002245509
84	IPI00216049	HNRNPK;MIR7-1 cDNA FLJ54552, highly similar to Heterogeneous nuclear ribonucleoprotein K	251	0.002210285
85	IPI00179330	UBB;RPS27A;UBC ubiquitin and ribosomal protein S27a precursor	250	0.002201479
86	IPI00418471	VIM Vimentin	247	0.002175062
87	IPI00008557	IGF2BP1 insulin-like growth factor 2 mRNA-binding protein 1 isoform 2	247	0.002175062
88	IPI00217030	RPS4X 40S ribosomal protein S4, X isoform	244	0.002148644
89	IPI00006181	EIF3D Putative uncharacterized protein EIF3D	240	0.00211342
90	IPI00011253	RPS3 40S ribosomal protein S3	238	0.002095808
91	IPI00550021	LOC653881;SNORD43;RPL3;SNORD83B 60S ribosomal protein L3 isoform b	237	0.002087002
92	IPI00008982	ALDH18A1 Isoform Long of Delta-1-pyrroline-5-carboxylate synthase	237	0.002087002
93	IPI00328328	SNORA63;MIR1248;SNORA4;SNORA81;SNORD2;EIF4A2 Isoform 2 of Eukaryotic initiation factor 4A-II	237	0.002087002
94	IPI00005024	MYBBP1A Isoform 2 of Myb-binding protein 1A	233	0.002051779
95	IPI00010153	RPL23 15 kDa protein	230	0.002025361
96	IPI00179298	HUWE1 Isoform 2 of E3 ubiquitin-protein ligase HUWE1	229	0.002016555
97	IPI00029012	EIF3A Eukaryotic translation initiation factor 3 subunit A	227	0.001998943
98	IPI00965012	TP53 Cellular tumor antigen p53	227	0.001998943
99	IPI00027280	TOP2B 134 kDa protein	225	0.001981331
100	IPI00000846	CHD4 Isoform 2 of Chromodomain-helicase-DNA-binding protein 4	225	0.001981331

Table 2. Phosphorylated E3 ligases identified by MS.

#	Protein	Protein names	Phospho (STY) Probabilities	Number of Phospho (STY)	Amino acid	Positions within proteins	Localization prob JK1	Intensity JK1
10	Q9UNE7	E3 ubiquitin-protein ligase CHIP	LGAGGGS(1)PEKSPSAQELK	1	S	19	0.999998	6971700
26	O95714	E3 ubiquitin-protein ligase HERC2	IRAEEEDLAAVPFLAS(0.998)DNEEEEDEKGNS	1	S	2928	0.998311	3244900
147	Q7Z6Z7	E3 ubiquitin-protein ligase HUWE1	ACS(0.998)PCS(0.001)SQSSSSGICTDFWDLLV	1	S	3373	0.998192	870010
226	Q7Z6Z7	E3 ubiquitin-protein ligase HUWE1	AQCET(0.047)LS(0.953)PDGLPEEQPQTTK	1	S	3662	0.952657	490320
257	Q7Z6Z7	E3 ubiquitin-protein ligase HUWE1	GS(0.001)GT(0.008)AS(0.991)DDEFENLR	1	S	1907	0.990735	364860

DISCUSSION

Each mechanism for IKKβ and STAT3 as well as their collaborative signaling have been extensively studied. However, the previous project was the first report to show that K63 -linked ubiquitination modification of the mutant IKKβ mediates the activation of STAT3 pathway. Thus, the follow-up project on investigating the mechanism of the signaling crosstalk had its importance in revealing the interactomes recruited by the scaffolding signal of the mutant IKKβ.

We utilized the proximity-dependent biotin identification method to find about 2500 interacting proteins of the IKKβ K171E 4KR. We carefully further screened for the proteins that were considered to be critical in activating the STAT3 pathway based on their known functions, and LRPPRC, IGF2BP1, and HUWE1 were chosen. We validated their involvement by using small interfering RNA to study the effect of the deficiency of each protein. siRNA-mediated LRPPRC knockdown showed slight increase in the activation of STAT3 caused by the mutant IKKβ. We do not know by which mechanism the LRPPRC protein plays a role in possibly inhibiting the constitutive inhibition of STAT3, however, LRPPRC forms a complex with Bcl-2 and Beclin1 which results in the inhibition the PI3K/Akt/mTOR pathway [20], which could otherwise activate the STAT3 [21]. siRNA-mediated inhibition of IGF2BP1 showed no effect on the activation of STAT3 by the mutant IKKβ. Similarly, siRNA-mediated knockdown of HUWE1 did not have an effect on either IL-6 mediated- or IKKβ mutant mediated- STAT3 activation.

Currently, we are investigating other E3 ligases that were identified to be highly phosphorylated from the Mass Spectrometry data from the BioID sample. CHIP and

HERC2 are shown to be more phosphorylated than HUWE1 although the total peptide abundance was significantly lower than that of HUWE1. However, they are still interesting candidates in that CHIP has shown to be catalyzing the K-63 linked ubiquitination of Hypoxia-Inducible Factor 1 α (HIF1 α). HIF1 α has an oncogenic role by promoting tumor cell adaptation to hypoxia and promoting angiogenesis. CHIP regulates degradation of HIF1 α through Chaperone-Mediated Autophagy by K-63 linked ubiquitination [22]. HERC2 is critical in the function of RNF8 E3 ligase which catalyzes K-63 linked ubiquitination for DNA damage repair signal to assemble BRCA1 and 53BP1 at sites of DNA breaks [23].

BioID method is advantageous in that it can screen for transient interactors or vicinal proteins and the biotin ligase fusion didn't seem to alter the biological effect of the protein of interest. However, biotin ligase itself could physically hinder the direct or indirect protein interactions. The result obtained, as the abundancy of biotinylated proteins doesn't necessarily correlate with the importance of the interaction, thus complementing the BioID method with proper validation approach will be of great guidance to screen for essential protein interactions.

We suggest that identifying the E3 ligase responsible for the signaling crosstalk with STAT3 can have a great clinical application. Although the mechanism could be a positive tumorigenic feedback loop by the increased cytokine production from the activated mutant IKKβ, or the ligase mediates the scaffolding signal of the mutant IKKβ to recruit other sets of proteins, inhibiting K-63 linked ubiquitination of the mutant IKKβ could lead to a positive outcome for the hematologic cancer patients.

MATERIALS AND METHODS

Plasmids

IKKβ WT 4KR (K301R, K418R, K555R and K703R) and IKKβ K171E 4KR plasmids were generated by Quikchange site-directed mutagenesis and confirmed by DNA sequencing. The IKKβ K171E 4KR construct was sub-cloned into mycBioID pcDNA3.1 using EcoRV and AflII site and also into BioID-HA tag pcDNA3.1 using AfeI and BsiWI site.

Cell culture for immune blot and siRNA analysis

HEK293T cells were grown in DMEM with 10% FBS and maintained in 10% CO_2 at 37°C. Cells were transfected with plasmid DNA using calcium phosphate precipitation at 3% CO_2.

BioID Cell Lines and Transfection.

HEK 293 cells were maintained at 10% CO_2 at 37˚C in DMEM supplemented with 10% FBS. The cells were transfected via calcium phosphate method and the transfected cells were subjected to G418 sulfate (Geneticin from Gibco, 0.5 mg/mL) selection for about 3 weeks while passing.

Affinity Capture of Biotinylated Proteins.

Two cell lines that stably expressing myBioID - IKKβ K171E 4KR and IKKβ K171E 4KR – BioID-HA were selected for analysis. Cells were incubated for 24h in

DMEM with 0.5mg/mL G418 sulfate and 50μM Biotin. Cells were treated with 10μM of MG132 ~4h prior to lysis. Cells were lysed at 25˚C in 1ml BioID lysis buffer. Triton X-100 was added to 2% final concentration. Cells were sonicated following the protocol [24]. Supernatants were incubated with 600 μL of streptavidin conjugated Dynabeads (Dynabeads® MyOne™ Streptavidin C1 from Invitrogen) overnight at 4˚C. Beads were collected and washed as in the protocol [24]. 10% of samples were reserved for Western Blot analysis. 90% of the samples were analyzed by mass spectrometry.

siRNA Transfection.

7×10^4 ~1.3×10^5 of HEK293T cells were plated into 6-well plates and incubated at 10% CO_2 at 37˚C in DMEM supplemented with 10% FBS for 24h. Cells were transfected with siHUWE1 or Non-targeting (Negative) siRNA (GE Dharmacon) using DharmaFECT reagent (GE Dharmacon) according to the manufacturer's instructions (Final concentration varied from 25nM to 75nM). The DMEM media was replaced 24h after the transfection to reduce cytotoxicity. 48h after the siRNA transfection, IKKβ K171E 4KR plasmid was transfected using calcium phosphate method and incubated at 3% CO_2 overnight. Cells were starved for 18h before harvest and lysis using RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS, 50mM NaF, 0.1mM PMSF, 10μg/ml Aprotinin, 1mM Na_3VO_4).

Antibodies and immunoblot

Antibodies of IKKβ (G-8), STAT3 (C-20), IGF2BP1 (D-9), and LRPPRC (H-300) were obtained from Santa Cruz Biotechnology; Phospho-STAT3 (Tyr705) (D3A7) from

Cell Signaling Technology; HUWE1 (A300-486A) from Bethyl Laboratories; Streptavidin Horseradish Peroxidase (HRP) Conjugate (Cat. 434323) from Invitrogen; horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit from GE Healthcare. Enhanced chemiluminence (ECL) reagents were from GE Healthcare. MG132 were obtained from Bio-techne; recombinant human Interleukin- (IL-6) from Life Technologies.

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