FIGURE LEGENDS

Figure 1 Effects of BAD BH3 stapled peptides on glucokinase (GK) activity. (**a**) GK activity in the presence of BAD $SAHB_A$ (S118D), BAD $SAHB_A$ (S118L), or vehicle alone. (**b**) Activation of GK by BAD $SAHB_A$ (S118D) in the presence or absence of the allosteric GK activator (GKA) RO0281675. Vertical lines in (**a**) and (**b**) indicate the glucose concentration corresponding to the $S_{0.5}$ values obtained from the representative experiment shown. Data in (**b**) show the means and range from a representative experiment, see **Table 1** for the summary of all independent experiments performed similarly (n = 3–18 per treatment).

Figure 2 Phospho-BAD BH3 peptides crosslink near the active site of GK. (**a**) Phospho-BAD BH3 peptides crosslink to GK after UV exposure in the presence or absence of glucose. See supplementary Fig. 1 for uncropped image of the gel. (**b**,**c**) Normalized spectral count frequency of BAD BH3 helix-crosslinked sites along the GK protein sequence in the presence of BAD $SAHB_A$ (Y110Bpa S118pS) (**b**) or BAD $SAHB_A$ (S118pS D123R F125Bpa). (**c**) Labeled residues are unique to each peptide and were found to occur ≥ 25% of the time. Protein sequences for the corresponding BAD SAHB peptide are included above each plot. Relevant substitutions to the wild-type sequence are denoted. (**d**,**e**) Mapping of BAD $SAHB_A$ (Y110Bpa S118pS) (**d**) or (S118pS D123R F125Bpa) (**e**) crosslinked residues onto surface view representations of the inactive super open, active intermediate, and active closed conformations of GK (PDB entries 1V4T[10], 4DCH[12] and 1V4S[10], respectively) locates the phospho-BAD BH3 binding interface to the enzyme's active site. To display an unobstructed view of the enzyme active site in the closed structure, the Active Site Loop (ASL) comprised of residues L164–N180 is presented in cartoon form rather than in surface view to show residues located behind the loop. Glucose is shown in green, allosteric GKAs in red, the ASL in white.

Figure 3 Mapping the phospho-BAD BH3 helix at the GK interaction site. (**a**–**c**) Phospho-BAD BH3 peptides bind to residues in the small domain of the inactive super open and the active intermediate, with the N-terminus localized around residue M115 and the C-terminus oriented toward residues V181, T206 and R333. The most frequently crosslinked residues for each BAD SAHB (≥25% threshold) are mapped onto the inactive super open (**a**), active intermediate (**b**), and active closed (**c**) conformers of GK. Residues selectively crosslinked by BAD $SAHB_A$ (Y110Bpa S118pS) and (S118pS D123R F125Bpa) are colored orange and blue, respectively, whereas residues crosslinked by both photoreactive BAD SAHBs are colored tan. For clarity, all of the associated residues are colored, but not all are labeled. Crosslinked residues common to both peptides are among those listed in Supplementary Table 3. Glucose is depicted in green, the Active Site Loop in white.

Figure 4 Effects of BAD BH3 stapled peptides on human islet function and human GK mutations. (**a**) Stimulation of insulin release by glucose in donor islets treated with BAD BH3 stapled peptides. Error bars represent means ± s.e.m. * $P < 0.05$, *** $P < 0.001$, n.s., non-significant; two-way ANOVA. (**b**,**c**) Effects of BAD $SAHB_A$ (S118D) on the activity of GK active site mutations. Kinetic analyses of two human GK mutants, M298K (**b**) and E300K (**c**), in the absence or presence of BAD $SAHB_A$ (S118D). Wild-type human GK treated with vehicle was included in each assay as a control. Vertical lines indicate the glucose concentration corresponding to the $S_{0.5}$ obtained from the representative experiment shown. The data in (**b**) and (**c**) show means and range from representative experiments, see **Table 2** for the summary of all independent experiments performed similarly (four experiments per GK mutant).

TABLES

Table 1 Modulation of glucokinase kinetic parameters by BAD BH3 stapled peptides

Treatment	$S_{0.5}$ (mM)	V_{max} (% veh.)	n_H	$S_{0.5\,Veh}/S_{0.5\,Treat}$	
Vehicle	7.49 ± 0.18	100	1.61 ± 0.03	1.00 ± 0.05	*n* = 18
GKA (RO0281675)	1.62 ± 0.13***	138 ± 6.15***	1.71 ± 0.06	4.62 ± 0.47***	*n* = 6
BAD $SAHB_A$ (S118D)	6.54 ± 0.15**	144 ± 4.64***	1.71 ± 0.03	1.15 ± 0.05	*n* = 18
BAD $SAHB_A$ (S118pS)	6.00 ± 0.68**	129 ± 6.54**	1.69 ± 0.18	1.25 ± 0.17	*n* = 3
BAD $SAHB_A$ (S118L)	8.14 ± 0.41	89.6 ± 5.01	1.62 ± 0.05	0.92 ± 0.07	*n* = 7
BAD $SAHB_A$ (S118D) + GKA	1.35 ± 0.07***	124 ± 1.54*	1.84 ± 0.07*	5.55 ± 0.41***	*n* = 4

Summary of the effects of BAD BH3 stapled peptides on GK activity. Kinetic parameters were derived using the Hill equation as described in Methods. $S_{0.5\ Veh}/S_{0.5\ Treat}$ in the fourth column calculates the fold change in glucose affinity resulting from each treatment. Data represent means ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with vehicle control (veh.); one-way ANOVA.

Table 2 Enhancement of GK activity by phospho-mimic BAD BH3 in select MODY2 mutations

Treatment	$S_{0.5}$ (mM)	V_{max} (% WT V_{max})	n_H	
GK (Wild-type) + Vehicle	5.60 ± 0.06	100	1.73 ± 0.03	*n* = 8
GK (M298K) + Vehicle	11.3 ± 0.90***	57.1 ± 4.02***	1.24 ± 0.05***	*n* = 4
GK (M298K) + BAD $SAHB_A$ (S118D)	8.08 ± 0.57†††	95.7 ± 9.47†††	1.43 ± 0.04†	*n* = 4
GK (E300K) + Vehicle	8.39 ± 0.12***	84.3 ± 1.89*	1.83 ± 0.03	*n* = 4
GK (E300K) + BAD $SAHB_A$ (S118D)	8.02 ± 0.38	103 ± 8.35†	2.11 ± 0.08††	*n* = 4

Summary of mutant GK activity in the absence or presence of BAD $SAHB_A$ (S118D). Kinetic parameters were derived using the Hill equation. Data represent means ± s.e.m. * $P < 0.05$, *** $P < 0.001$ compared with vehicle treated recombinant human wild-type (WT) GK; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared with vehicle treated mutant GK; one-way ANOVA.

METHODS

Recombinant GK expression and purification. Mouse GK isoform 1 was cloned in the pET-43.1 Ek/LIC vector (Novagen) containing an N-terminal hexahistidine tag that was further engineered to express a TEV cleavage site (ENLYFQS) preceding the GK sequence. The resultant recombinant GK was purified as follows and used in experiments documented in Fig. 1, 2, and 3, and Table 1, and Supplementary Tables 2 and 3. Briefly, *Escherichia coli* BL21(DE3) pLysS transformed with the construct were grown at 37 ^{o}C to an optical density of 0.5-0.6 at 595 nm, and the expression of GK fusion protein was induced with 0.6 mM IPTG for 6 h at 30 ^{o}C. The cells were harvested, resuspended in 100 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5 mM BME, 1 mM PMSF and EDTA-free complete protease inhibitor cocktail (Roche), and sonicated at 4 ^{o}C (Misonix, Inc. S-4000). The lysates were passed over a Ni-NTA agarose matrix (Qiagen) followed by ion-exchange chromatography with a Mono Q 5/50 GL (GE Life Sciences) using a NaCl gradient and gel filtration using a Superdex 75 10/300 GL column (GE Life Sciences) in 50 mM Tris, 150 mM, 5 mM DTT, pH 7.4. Aliquots of purified GK were stored at -80 ^{o}C with 20% glycerol (v/v).

N-terminal GST-tagged constructs (pGEX3) expressing the recombinant human wild-type GK isoform 1 and the M298K and E300K GK active site mutations used in Fig. 4 and Table 2 have been previously described[37,38]. Briefly, BL21(DE3) transformed with the constructs were induced with IPTG overnight at 25 ^{o}C, lysed in buffer containing 4 mM KH_2PO_4, 16 mM K_2HPO_4, 150 mM KCl and 5 mM DTT as previously described[38]. Tagged GST-hGK was captured using glutathione agarose beads (Sigma), cleaved on-column by incubation with Factor Xa (GE Life Sciences), and subjected to gel filtration.

SAHB synthesis. Peptide synthesis, olefin metathesis, amino terminal derivatization (e.g. biotin-βAla), reverse phase HPLC purification, and amino acid analysis were performed as previously described[22,33].

Steady-state kinetics. Glucokinase activity was measured by monitoring the rate of NADH formation using a G6PDH-coupled reaction as previously described[38], with minor modifications. Briefly, assays were performed at 37 ^{o}C in 100 μL total volume per well of a Costar 3596 plate using a SpectraMax M5 microplate reader (Molecular Devices). Absorbance at 340 nm was recorded every two min and the change in absorbance per min was calculated using data between 30 and 60 min, where the rate of reaction was linear. Reaction wells were prepared on ice, and contained final concentrations of 7.5 nM GK, 100 mM HEPES pH 7.4, 150 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 1 mM NAD, 0.05% BSA, 2.5 units G6PDH and 5% DMSO in the presence or absence of 5 μM BAD SAHBs (a concentration deemed near-saturating based on dose-response assays) and/or 3 μM of RO0281675 (http://www.axonmedchem.com). To determine glucose-dependent kinetic parameters, glucose was varied (0–50 mM) while maintaining ATP at 5 mM. Kinetic parameters were derived from the Hill equation using GraphPad Prism 6.0a.

Crosslinking of photoreactive BAD SAHBs and glucokinase. Photoreactive BAD SAHBs were applied in protein target crosslinking as previously reported[33]. Briefly, SAHBs (10 μM) containing benzophenone moieties were mixed with recombinant mouse glucokinase (5 μM) in buffer A (50 mM Tris, 5mM DTT, 200 mM NaCl, pH 7.5) with or without the addition of 50 mM glucose as indicated, incubated for 15 min, and then irradiated with UV light (365 nm) for 2 h on ice. Unreacted SAHBs were removed by dialysis overnight at 4 ^{o}C in buffer B (50mM Tris, 200mM NaCl, pH 7.5) using 6-8 kDa MWCO D-tube dialyzers (EMD Biosciences). Crosslinking

in the presence of RO0281675 was performed with 50 mM glucose and 3 μM RO0281675. Biotin affinity capture, elution, and gel electrophoresis were performed as described[33].

Western analysis of photoaffinity captured proteins. Following electrophoresis and transfer to PDVF, membranes were blocked in PBS containing 5% milk for 30 min at room temperature. The membranes were rinsed with PBS containing 3% bovine serum albumin (BSA), and subsequently probed with purified goat anti-biotin antibody conjugated to horseradish peroxidase (Cell Signaling Technology, #7075) at a dilution of 1:1000 in PBS containing 3% BSA overnight as previously described[33]. After washing 3 times for 15 min with PBS containing 0.1% Tween-20, bands were detected by chemiluminescence (PerkinElmer).

Mass spectrometry analysis. Samples were subjected to in-gel digestion and mass spectrometry as described[33]. Searches were done using the sequest algorithm[53], with a sequence-reversed search database comprised of GK, trypsin, and common keratin contaminants. Search results were limited to those peptides containing a BAD SAHB crosslinked adduct. False positive identifications (considered to be any crosslinked peptides derived from trypsin or keratins, from the reversed database, or peptides with masses corresponding to more than one crosslinked adduct) were limited to less than 5% via linear discriminant analysis of multiple variables including mass accuracy, XCorr, tryptic state, and charge state. MS/MS spectral counting was performed as described[33]. Spectral counts for crosslinking to each residue were normalized in each experiment such that the most frequently detected residue was assigned a value of 100%, while 0% indicated no crosslinking. The normalized values for each experiment were then combined and averaged to generate frequency distribution plots. For Fig. 3, a value of 25% for this normalized average frequency of spectral counts was chosen for depicting the differential crosslinking pattern of N- and C-terminal photoreactive BAD SAHBs. This 25% cutoff was chosen in accordance with

observations from both published[26,33] and unreported experiments that when crystal structures of an interaction are known, higher relative crosslinking frequency generally correlates with better agreement with these structures.

Human islets culture, SAHB treatment and insulin release assays. Human donor islets were obtained through the Integrated Islet Distributing Program (http://iidp.coh.org/). Islets were cultured according established protocols[54], at 37 ^{o}C (5% CO_2) in CMRL 1066 media (Gibco) containing 5.5 mM glucose and supplemented with human albumin serum (10%, Gemini)[55], L-Glutamine (2 mM, Sigma) and HEPES (1 mM, Sigma), and 0.1% insulin-transferrin-selenium, pH 7.4. Islets were cultured at least an additional 24 h prior to treatment with 3 μM BAD SAHBs or vehicle (DMSO 0.3%) in CMRL 1066 medium containing 5.5 mM glucose, supplemented with 10% human serum (pH 6.2), overnight at 37 ^{o}C. Islets were then incubated for 90 min in basal conditions (1.67 mM glucose), hand picked and incubated in either basal (1.67 mM glucose) or stimulatory glucose condition (20 mM glucose) for 45 min[54], and insulin secretion was measured as previously described[56].

Statistical analysis. Statistical significance was calculated in GraphPad Prism 6.0a (www.graphpad.com) using one-way ANOVA followed by an appropriate post-test. When comparing the effects of BAD SAHB compounds on wild-type GK activity (Table 1 and Supplementary Table 2), comparisons were made using one-way ANOVA followed by Dunnett's test using the vehicle data as the control in each case. When comparing mutant GK activity in the absence or presence of BAD $SAHB_A$ (S118D) (Table 2), analyses used one-way ANOVA followed by a Holm-Šídák test involving four comparisons in total. When comparing the effects of BAD SAHB compounds on insulin secretion in human islets, two-way ANOVA was used followed by a Tukey multiple comparisons test. For all analyses, thresholds for significance used multiplicity adjusted *P* values to account for multiple comparisons.

53. Eng, J.K., McCormack, A.L. & Yates, J.R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**, 976-989 (1994).

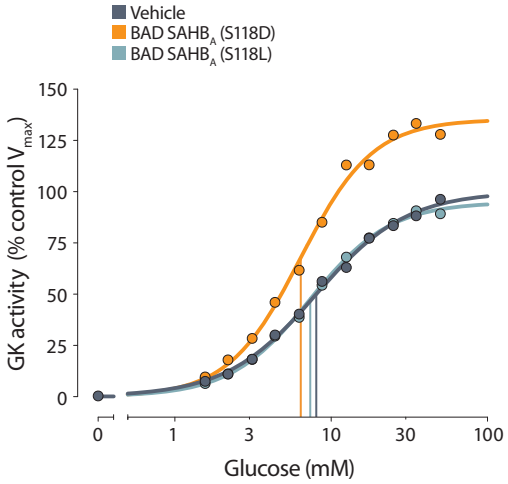
54. Murdoch, T.B., McGhee-Wilson, D., Shapiro, A.M. & Lakey, J.R. Methods of human islet culture for transplantation. *Cell Transplant* **13**, 605-617 (2004).

55. Barbaro, B.*, et al.* Increased albumin concentration reduces apoptosis and improves functionality of human islets. *Artif Cells Blood Substit Immobil Biotechnol* **36**, 74-81 (2008).

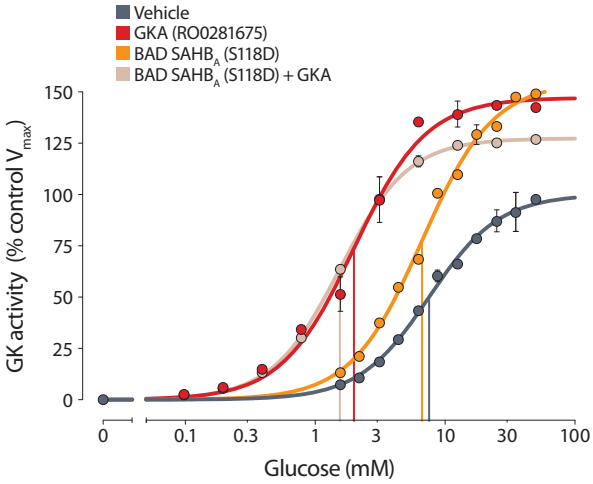
56. Mahdi, T.*, et al.* Secreted frizzled-related protein 4 reduces insulin secretion and is overexpressed in type 2 diabetes. *Cell Metab* **16**, 625-633 (2012).

Figure 1

a


Vehicle
BAD SAHB$_A$ (S118D)
BAD SAHB$_A$ (S118L)
GK activity (% control V_{max})
150
125
100
75
50
25
0
0
1
3
10
30
100
Glucose (mM)

b


Vehicle
GKA (RO0281675)
BAD SAHB$_A$ (S118D)
BAD SAHB$_A$ (S118D) + GKA
GK activity (% control V_{max})
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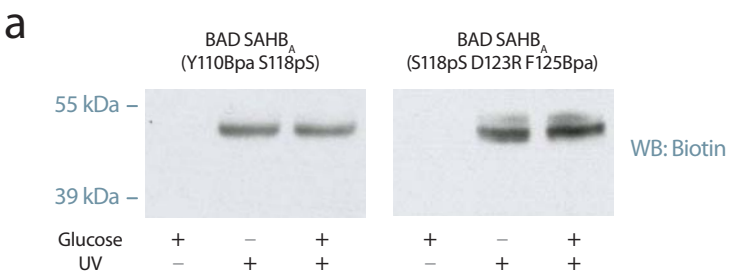
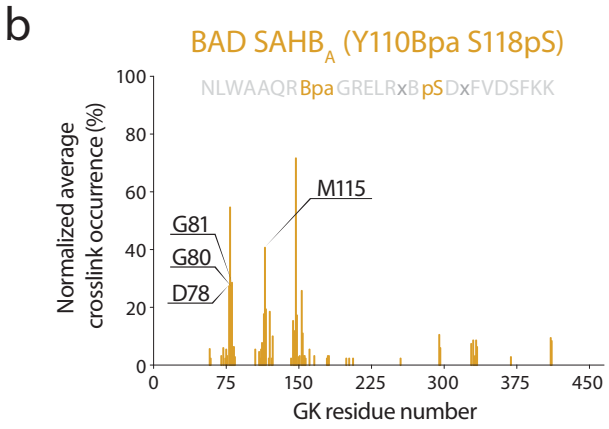
125
100
75
50
25
0
0
0.1
0.3
1
3
10
30
100
Glucose (mM)

Figure 2


a
BAD SAHB$_A$ (Y110Bpa S118pS)
BAD SAHB$_A$ (S118pS D123R F125Bpa)
55 kDa –
39 kDa –
WB: Biotin
Glucose + – + + – +
UV – + + – + +


b
BAD SAHB$_A$ (Y110Bpa S118pS)
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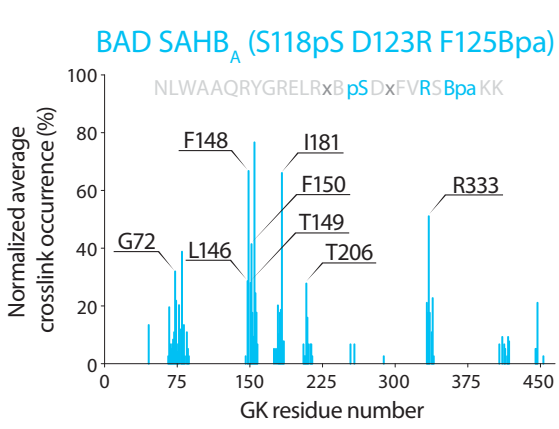
Normalized average crosslink occurrence (%)
M115
G81
G80
D78
GK residue number


c
BAD SAHB$_A$ (S118pS D123R F125Bpa)
NLWAAQRYGRELRxBpSDxFVRSBpaKK
Normalized average crosslink occurrence (%)
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I181
F150
R333
T149
G72
L146
T206
GK residue number

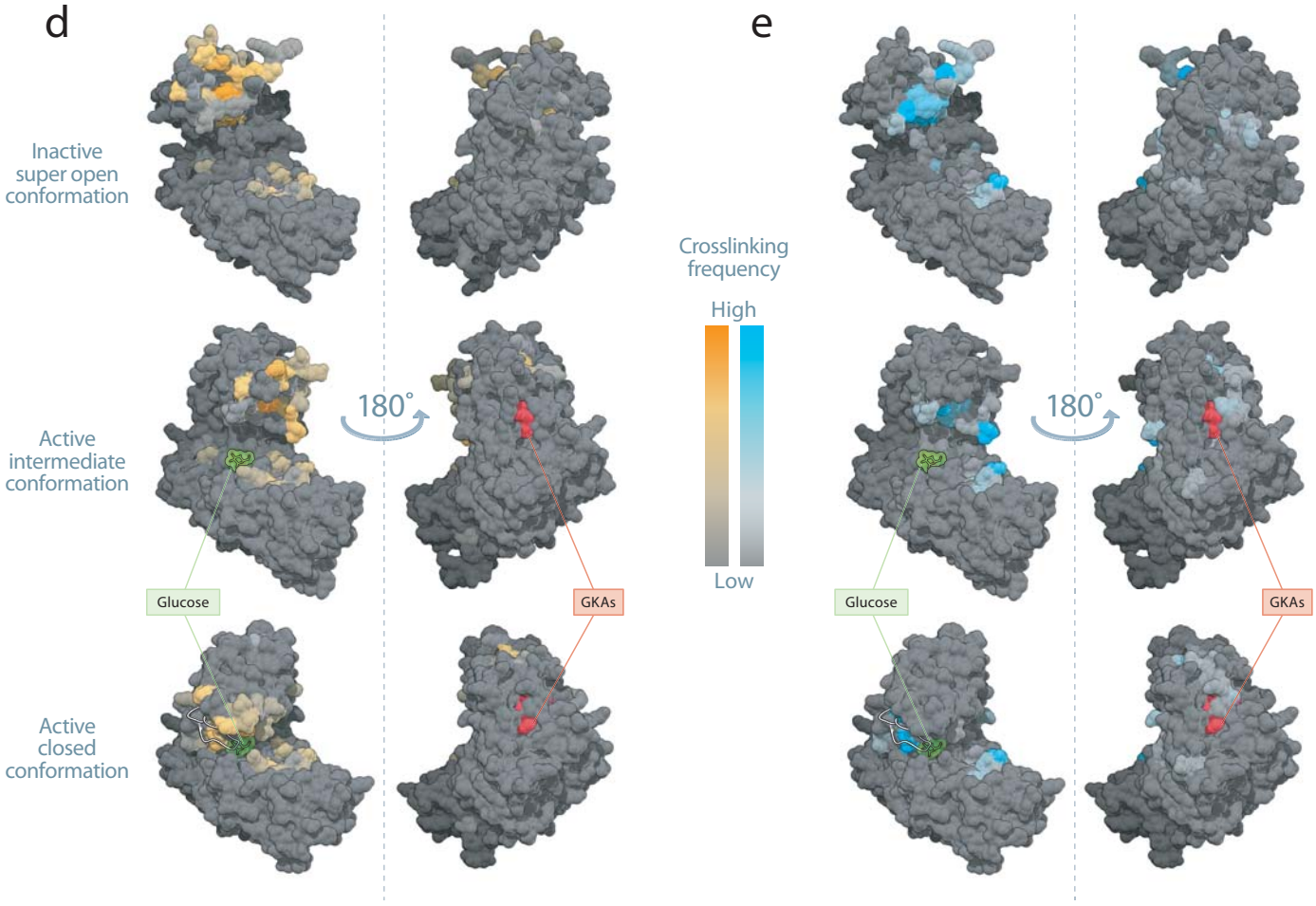

d
e
Inactive super open conformation
Active intermediate conformation
Active closed conformation
180˚
Glucose
GKAs
Crosslinking frequency
High
Low

Figure 3

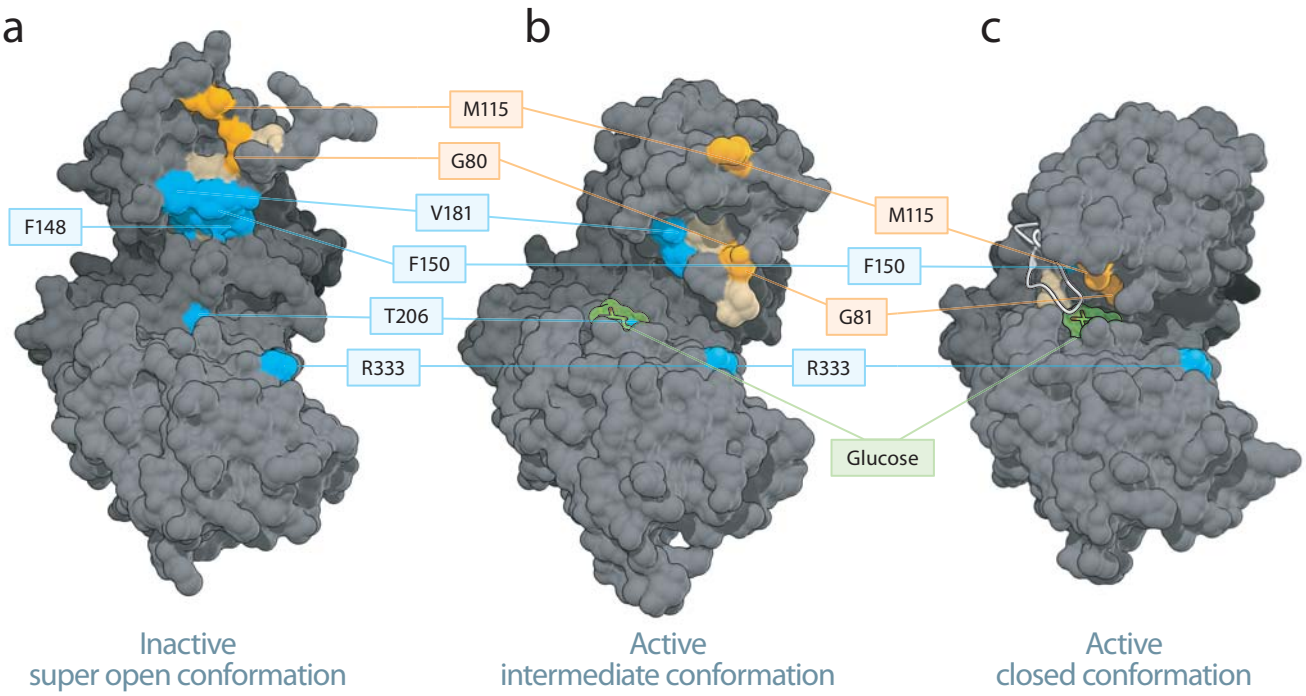
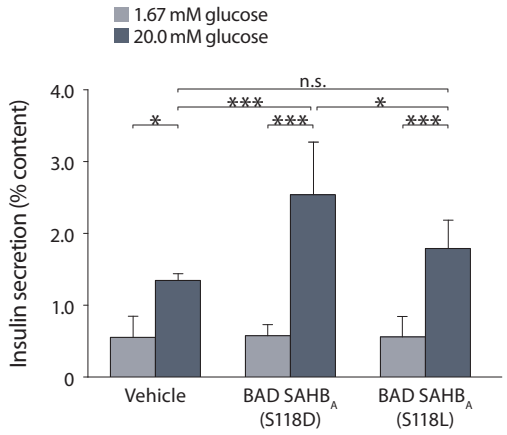
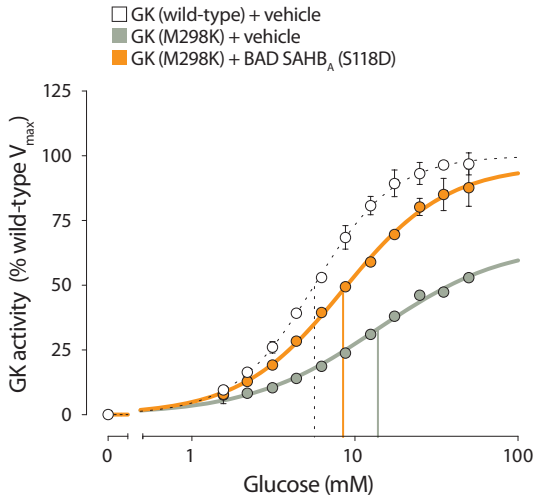

a
b
c
M115
G80
V181
F148
F150
T206
R333
M115
F150
G81
R333
Glucose
Inactive
super open conformation
Active
intermediate conformation
Active
closed conformation

Figure 4

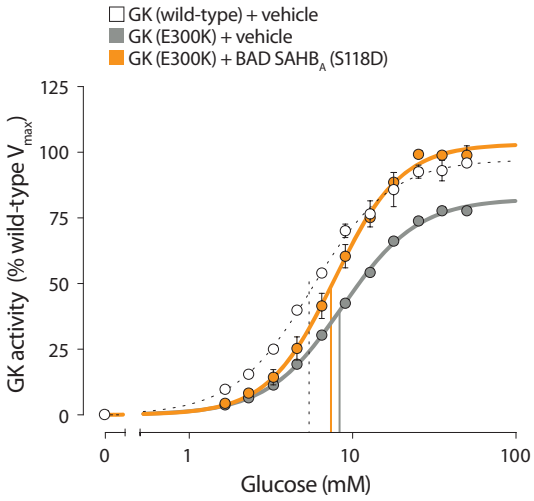
a

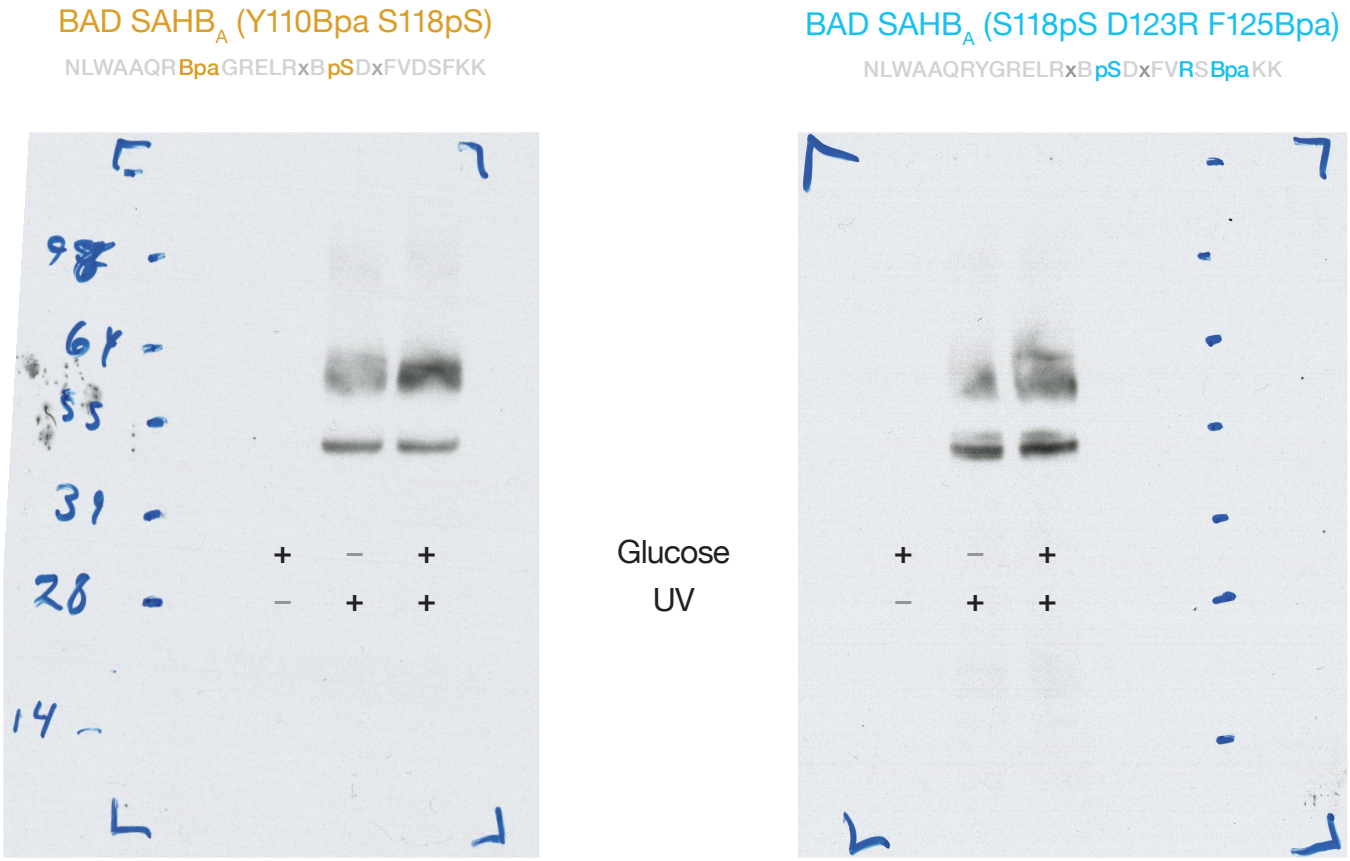

1.67 mM glucose
20.0 mM glucose
Insulin secretion (% content)
4.0
3.0
2.0
1.0
0
n.s.
***
*
*
***
***
Vehicle
BAD SAHB$_A$ (S118D)
BAD SAHB$_A$ (S118L)

b

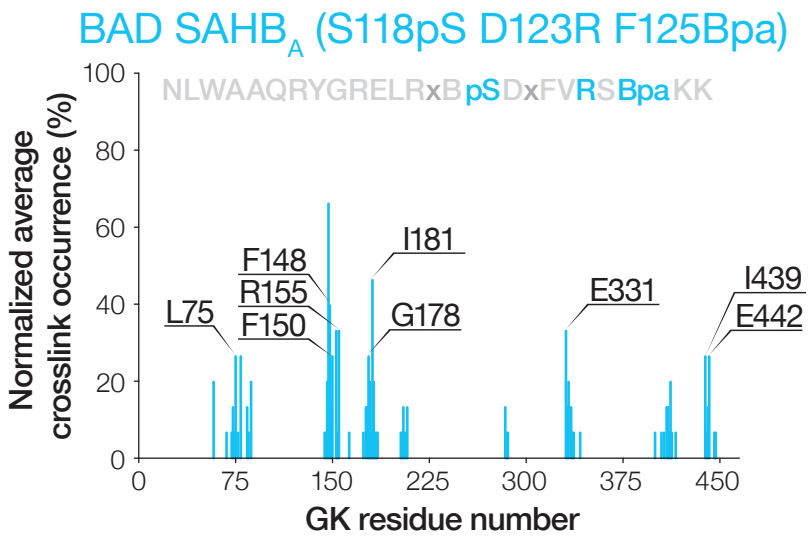

GK (wild-type) + vehicle
GK (M298K) + vehicle
GK (M298K) + BAD SAHB$_A$ (S118D)
GK activity (% wild-type V$_{max}$)
125
100
75
50
25
0
0
1
10
100
Glucose (mM)

c


GK (wild-type) + vehicle
GK (E300K) + vehicle
GK (E300K) + BAD SAHB$_A$ (S118D)
GK activity (% wild-type V$_{max}$)
125
100
75
50
25
0
0
1
10
100
Glucose (mM)

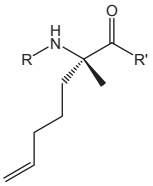


Supplementary Figure 1. Phospho-BAD BH3 peptides crosslink to GK after UV exposure in the presence or absence of glucose. Uncropped gels related to Fig. 2a document detection of BAD SAHB$_A$–GK crosslinked complex as visualized by western blot analysis using an anti-biotin antibody. The lower band corresponds to monomeric GK crosslinked to the BAD SAHB$_A$ compound, which was excised and subjected to MS/MS analysis.

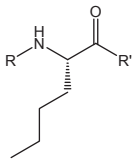


Supplementary Figure 2. Crosslinking profile of BAD BH3 stapled peptide in the presence of glucose and RO0281675. Spectral count frequency of BAD SAHB$_A$ (S118pS D123R F125Bpa) crosslinked along the GK protein sequence. GK residues involved in crosslinking, which were unique to that peptide (with a frequency >25%), are labeled.

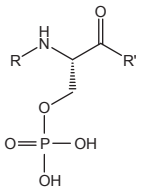
Peptide	N-term	Sequence	Applied in
BAD SAHB$_A$ (S118D)		NLWAAQRYGRELRXBDDXFVDSFKK	Fig. 1, 4, Table 1, 2
BAD SAHB$_A$ (S118L)		NLWAAQRYGRELRXBLDXFVDSFKK	Fig. 1, 4, Table 1
BAD SAHB$_A$ (S118pS)		NLWAAQRYGRELRXBpSDXFVDSFKK	Table 1
BAD SAHB$_A$ (Y110Bpa S118pS)	Biotin-βala	NLWAAQRUGRELRXBpSDXFVDSFKK	Fig. 2, 3, Supp. Fig. 1, Supp. Table 2, 3
BAD SAHB$_A$ (S118pS D123R F125Bpa)	Biotin-βala	NLWAAQRYGRELRXBpSDXFVRSUKK	Fig. 2, 3, Supp. Fig. 1, 2, Supp. Table 2, 3
BAD SAHB$_A$ (S118D)	Biotin-βala	NLWAAQRYGRELRXBDDXFVDSFKK	Supp. Table 2



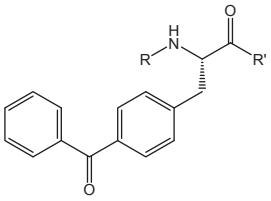
X : (S)-2-(4'-pentenyl) alanine



B : L-norleucine



pS : phosphoserine



U : 4-benzoyl-L-phenylalanine (Bpa)

Supplementary Table 1. Composition of BAD BH3 stapled peptides. BAD BH3 compounds were synthesized as described in Methods. The N-termini of the peptides were acetylated unless otherwise stated. Residues in the protein sequence highlighted in green indicate substitutions from the wild-type sequence, while those in red denote changes to non-natural amino acids.

Treatment	$S_{0.5}$ (mM)	V_{max} (% veh.)	n_H	
Vehicle	8.35 ± 0.04	100	1.59 ± 0.03	(n = 3)
BAD $SAHB_A$ (S118D)	7.06 ± 0.42	144 ± 11.8 *	1.70 ± 0.06	(n = 3)
BAD $SAHB_A$ (Y110Bpa S118pS)	6.29 ± 0.34 *	152 ± 14.5 *	1.83 ± 0.06 *	(n = 3)
BAD $SAHB_A$ (S118pS D123R F125Bpa)	6.92 ± 0.51	122 ± 8.80	1.71 ± 0.06	(n = 3)

Supplementary Table 2. Summary of the effects of biotinylated BAD BH3 stapled peptides. Kinetic parameters were derived using the Hill equation as described in Methods. Data represent means ± s.e.m. * $P < 0.05$ compared with vehicle control, one-way ANOVA.

a

BAD $SAHB_A$ (Y110Bpa S118pS)

GK residue	Mean frequency
G147	72%
L79	55%
M115	41%
G81	29%
D78	27%
G80	26%
P153	26%
T116	19%
A114	18%
E120	18%

b

BAD $SAHB_A$ (S118pS D123R F125Bpa)

GK residue	Mean frequency
P153	76%
G147	67%
I181	66%
F148	60%
R333	56%
F150	41%
L79	39%
G72	32%
L146	29%
T206	28%

Supplementary Table 3. GK residues most frequently involved in crosslinking with BAD BH3 stapled peptides. Normalized average frequency of phospho-BAD BH3 peptides derivitized with a 4-benzoyl-L-phenylalanine at either the N-terminus (**a**) or C-terminus (**b**).