Supporting Information

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SI Text

The analytical data is as follows: (-)-para-azido-blebbistatin: ^{1}H chemical shifts, ppm: 2.26 (2, 2H, multiplet), 2.30 (11, 3H, singlet), 3.95 (3A, 1H, multiplet), 4.05 (3B, 1H, multiplet), 6.84 (12, 1H, singlet), 7.12 (8, 1H, doublet, $^{3}J_{HH}$ = 8.1 Hz), 7.19 (3′, 2H, doublet, $^{3}J_{HH}$ = 8.9 Hz), 7.40 (7, 1H, dd, $^{3}J_{HH}$ = 8.1Hz, $^{4}J_{HH}$ = 1.8 Hz), 7.54 (5, 1H, doublet, $^{4}J_{HH}$ = 1.8 Hz), 8.15 (2′, 2H, doublet, $^{3}J_{HH}$ = 8.9 Hz). ^{13}C chemical shifts, ppm: 20.0 (11), 28.0 (2), 47.2 (3), 73 (3A), 119.1 (3′), 120.8 (2′), 121.1 (10), 125.5 (8), 126.0 (5), 132.4 (6), 134.4 (1′), 136.0 (7), 138.1 (4′), 148.9 (9), 165.4 (2x), 194.7 (4). ES-MS (Q-TOF Premier mass spectrometer; Waters): (M+1) 334.1306. Calculated for $C_{18}H_{16}N_5O_2$, 334.1304. Brownish yellow powder. Storage: −20 °C. Protect from light. No degradation in 1 y.

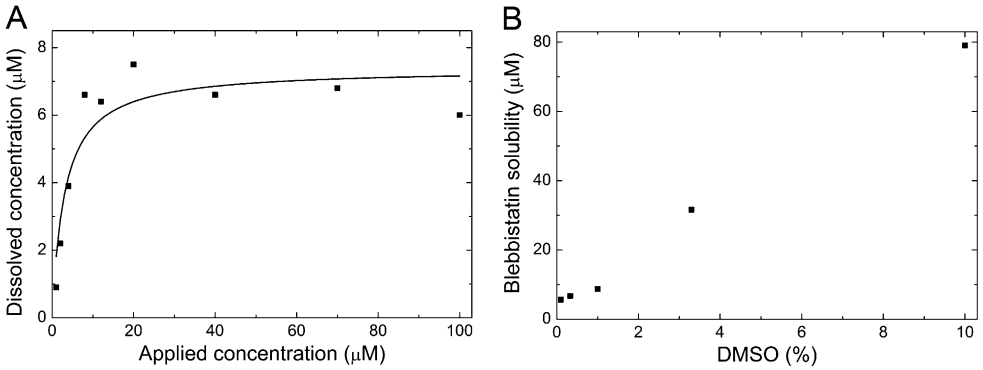

Fig. S1. Solubility of blebbistatin in assay buffer and its dependence on DMSO concentration. (*A*) 1, 2, 4, 8, 12, 20, 40, 70, and 100 μM blebbistatin solutions in assay buffer were prepared, vortexed thoroughly, and centrifuged at 12,100 × *g* for 5 min. Absorption spectra of the supernatants were recorded and the concentrations of the dissolved blebbistatin were determined ($\varepsilon^{\circ}_{427}$ = 6,100 $M^{-1}\cdot cm^{-1}$). Based on the fitted hyperbola, the solubility of blebbistatin in assay buffer (see *Materials and Methods*) containing 0.1% DMSO was 7.4 ± 0.6 μM. (*B*) Next, 100 μM blebbistatin solutions were prepared in assay buffer containing 0.1%, 0.3%, 1%, 3.3%, and 10% DMSO. After vortexing, the solutions were centrifuged at 12,100 × *g* for 5 min. Absorption spectra of the supernatants were recorded and the concentrations of dissolved blebbistatin were determined. The solubility of blebbistatin increased quasi-linearly with DMSO concentration to 80 μM at 10% DMSO.

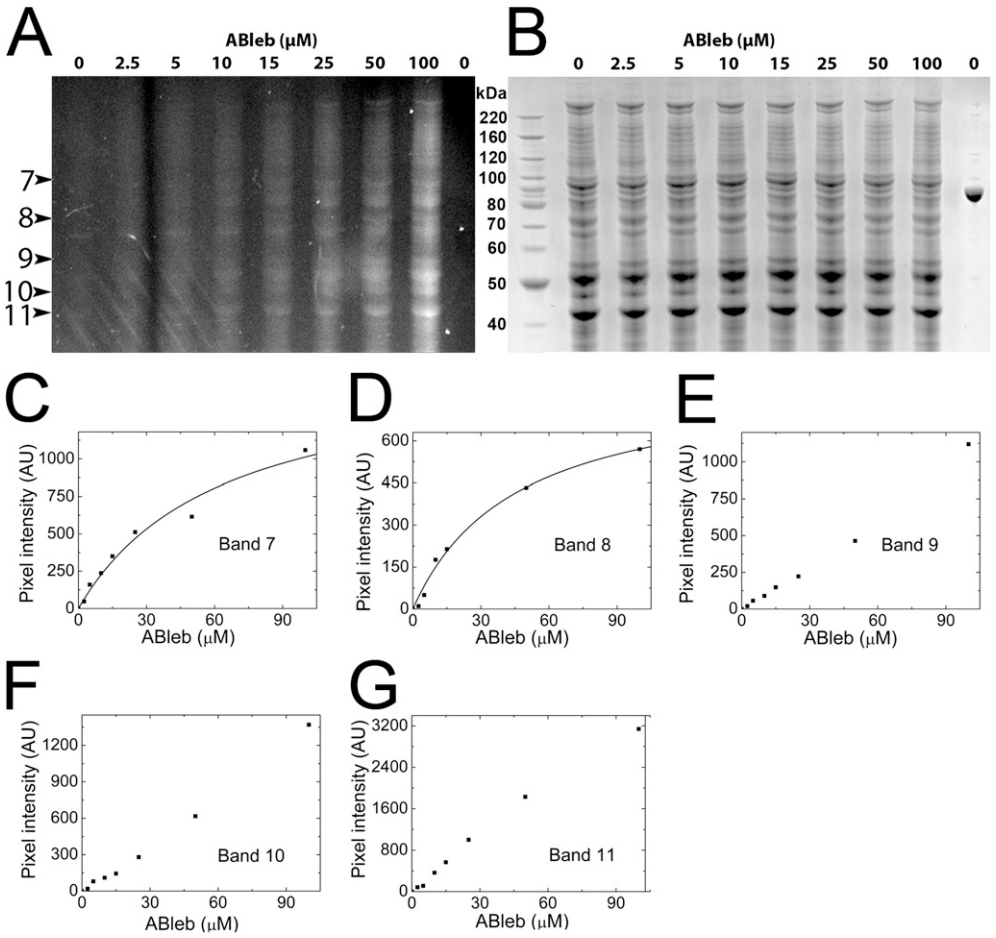

Fig. S2. SDS/PAGE analysis of azidoblebbistatin crosslinking of proteins in *Dictyostelium discoideum* (*Dd*) whole-cell lysates. Azidoblebbistatin-attached proteins in *Dd* myosin II motor domain (*DdMd*)-expressing *Dd* whole-cell lysates were detected by fluorescence of the covalently bound inhibitor (*A*) and, on the same gel, the protein contents were analyzed by subsequent Coomassie staining (*B*). Purified *DdMd* was loaded in the right-most lane of the gel as a control. Fluorescent bands are indicated at the left side of panel *A* in the order of increasing mobility. (*C*–*G*) The azidoblebbistatin concentration dependence of the fluorescence intensity of azidoblebbistatin-crosslinked protein bands. Determined EC_{50} values were 63 μM and 45 μM for bands 7 and 8, respectively. In the case of bands 9–11, the EC_{50} values were higher than 100 μM.