

# SUPPORTING INFORMATION

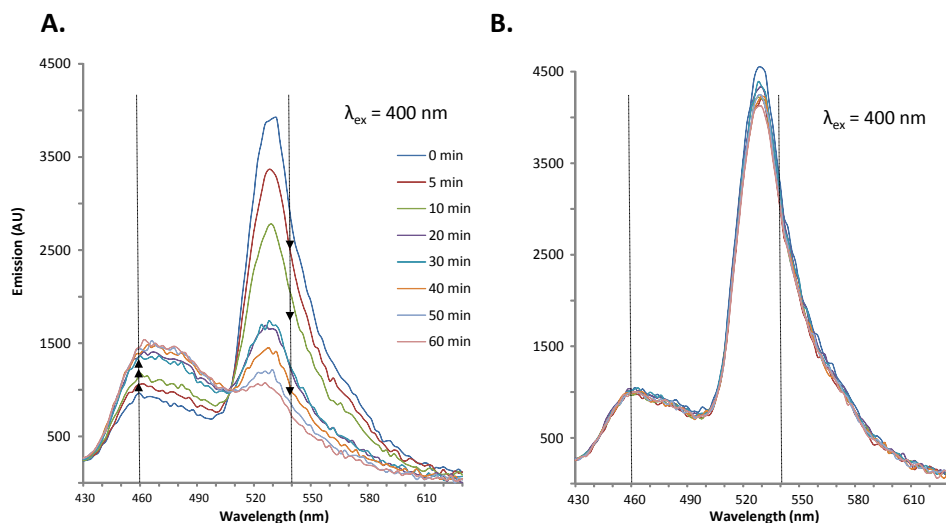
## **Optical assay of hedgehog protein cholesterololysis**

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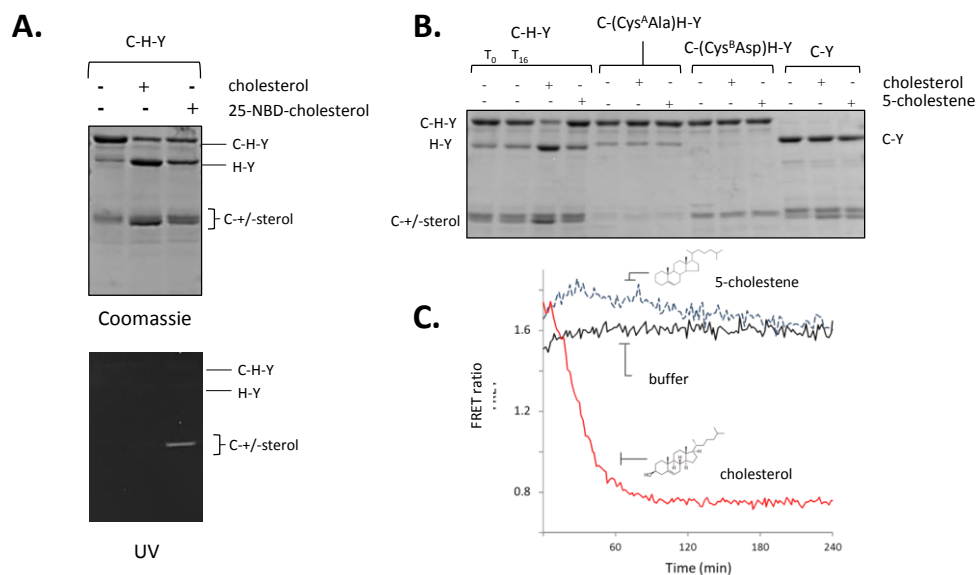
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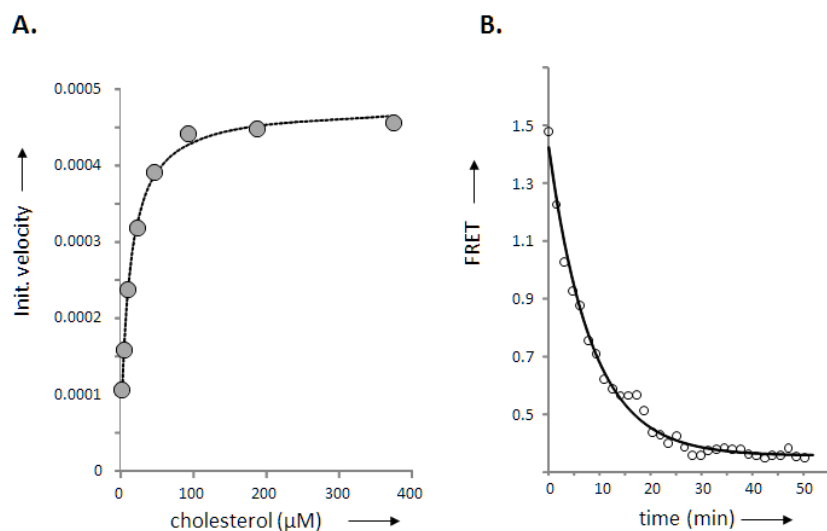
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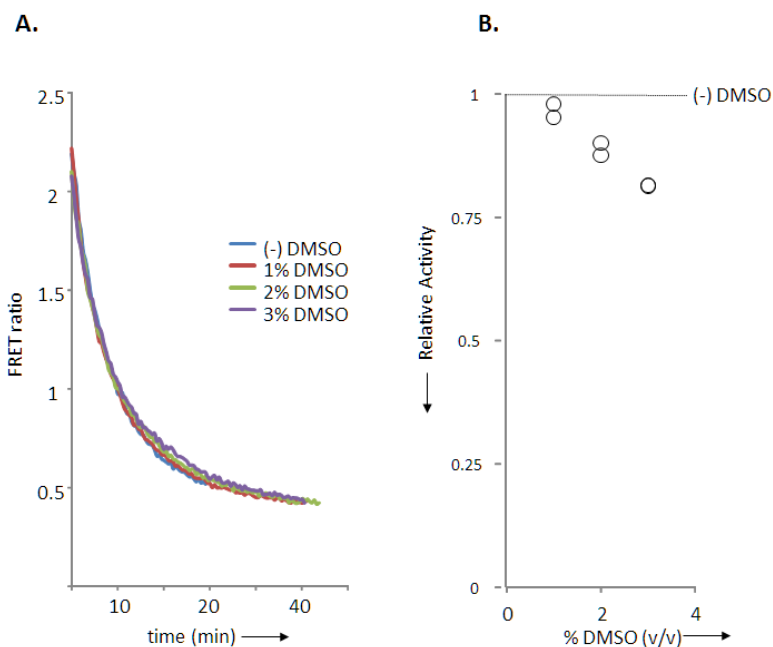
**Supporting Figure 1.** Time-resolved fluorescence spectra of C-H-Y undergoing cholesterolysis. Sample excitation wavelength is 400 nm. Hashed lines indicate donor emission ( $\lambda_{em}$ , 460 nm) and acceptor emission ( $\lambda_{em}$ , 540 nm) wavelengths used in calculating FRET ratio. Experiments were conducted in the presence of native substrate cholesterol (A), and an unreactive substrate analogue, 5-cholestene (B).



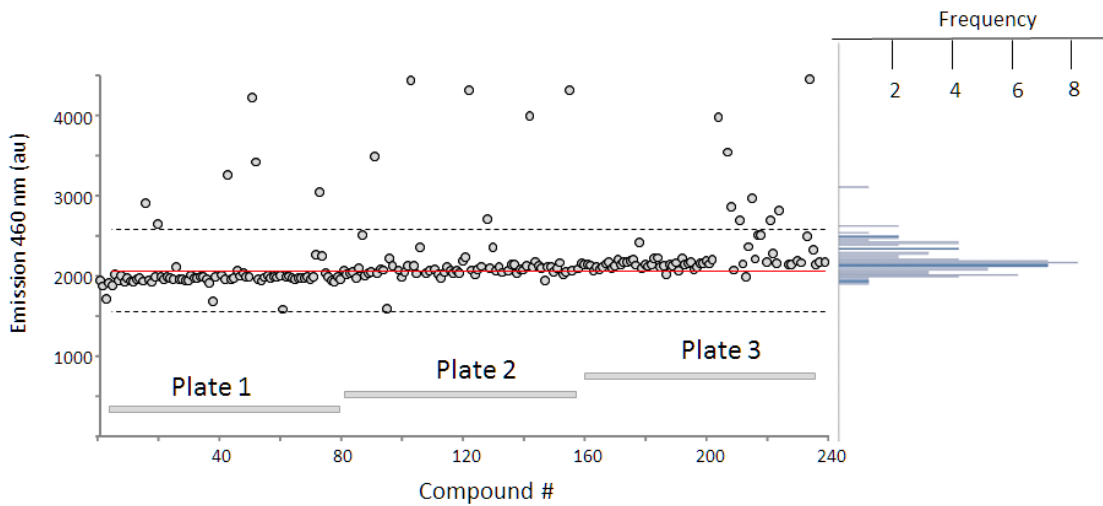
**Supporting Figure 2.** Assay validation using catalytic mutants and substrate analogues. (A) Evidence for covalent modification by using 25-NBD cholesterol. C-H-Y was incubated in BENTT buffer +/- cholesterol or 25-NBD cholesterol for 18 h, separated by denaturing SDS-PAGE, and imaged with coomassie blue and UV. (B) Activity of C-H-Y toward cholesterol but not 5-cholestene. C-H-Y and control constructs were incubated in BENTT buffer +/- sterol for 18 h, separated by denaturing SDS-PAGE, and stained with coomassie blue. (C) FRET assay comparing substrate activity cholesterol and 5-cholestene. Kinetic monitoring of FRET signal from C-H-Y in the presence of 5-cholestene, buffer, and cholesterol over a period of 4 h.



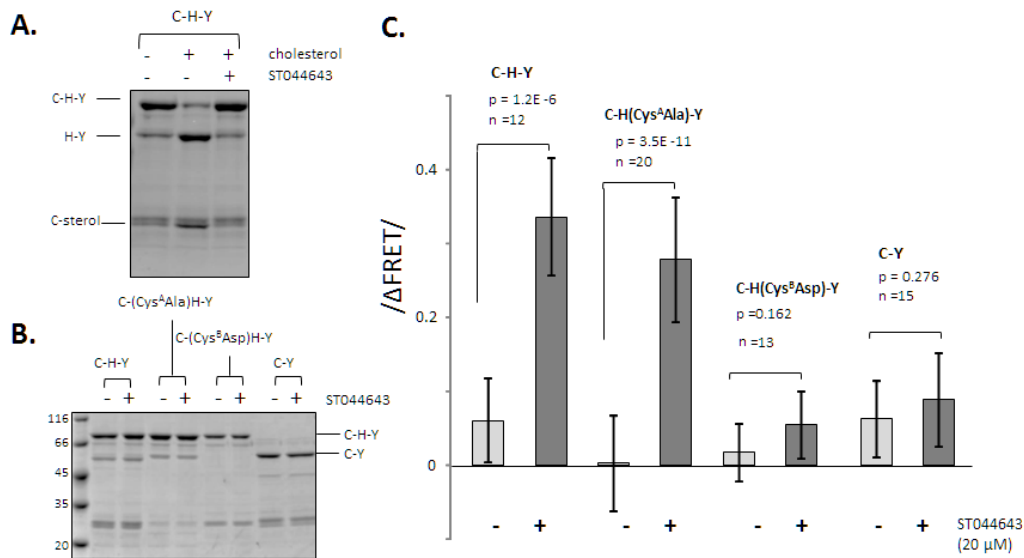
**Supporting Figure 3.** Kinetics of Hh cholesterololysis. (A) Initial rates of cholesterololysis, as measured by the decreasing FRET from C-H-Y, plotted as function of increasing concentration of cholesterol. Trend line is calculated using the Michaelis Menten equation with a  $K_m$  value of 15  $\mu\text{M}$ . (B) Maximum cholesterololysis rate. Declining FRET signal accompanying the consumption of C-H-Y in the presence of saturating cholesterol followed a first order exponential decay ( $\Delta\text{FRET} = A * e^{-kt} + C$ ). Trend line shows the behavior expected for a first order process with rate constant of  $0.001 \text{ sec}^{-1}$ .



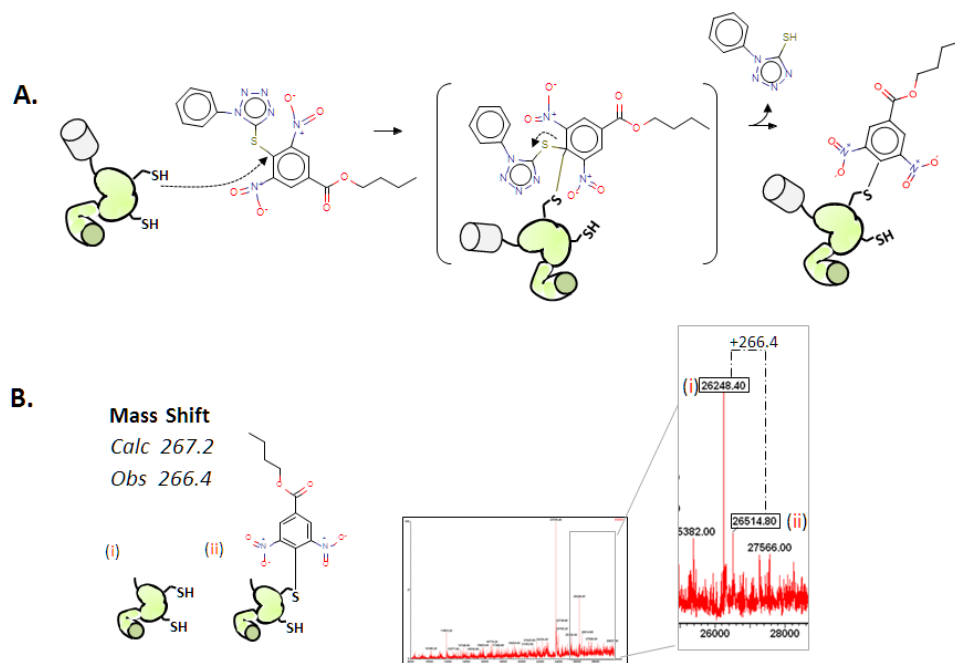
**Supporting Figure 4.** Sensitivity of C-H-Y kinetics to added DMSO. (A) Representative traces of C-H-Y samples in the presence of the indicated % of DMSO (v/v). Initial cholesterol concentration was 250  $\mu\text{M}$ . (B) Relative cholesterololysis activity of C-H-Y plotted as a function of DMSO. Apparent rate constants of cholesterololysis in the presence of (+) DMSO were obtained by fitting kinetic traces to first order exponentials, as above; values were then divided by the rate constant for a control reaction carried out in the absence of DMSO to obtain a measure of fractional activity, plotted on the y axis.



**Supporting Figure 5.** Identifying optically active compounds. Selected compounds (8% of total) from the TimTec library showed fluorescence (ex 400 nm, em 460 nm) that exceeded the average fluorescence from control wells lacking compound (red line, and histogram) by +/- three standard deviations (dotted lines). Data from assay wells containing these outliers were not analyzed further.



**Supporting Figure 6.** Interaction of ST044643 with HhC. (A) Inhibition of cholesterololysis. C-H-Y activity was monitored by denaturing SDS-PAGE in buffer (left lane), in buffer containing cholesterol (middle lane), and in buffer containing cholesterol with added ST044643 (right lane). (B) Stability of C-H-Y and derivatives +/- ST044643. Indicated constructs (2  $\mu$ M) were incubated with ST044643 (20  $\mu$ M) for 1 h, followed by denaturing SDS-PAGE. (C) FRET quench binding assay. Observed changes in FRET from C-Y, C-H-Y, C-(Cys<sup>A</sup>Ala)H-Y, and C-(Cys<sup>B</sup>Asp)H-Y in BENT buffer at 30 °C +/- added ST044643 (20  $\mu$ M). Data were collected after fluorescence readings had reached a stable value, usually 30 min. Error bars indicate standard deviation.



**Supporting Figure 7.** Proposed inactivation of Hh by ST044643. (A)  $S_nAr$  mechanism involves the initial attack by conserved active site cysteine residue (CysB) (left), generating an unstable Meisenheimer intermediate (middle) that resolves by displacement of the mercaptotetrazole moiety (right). (B) Detection of covalent adduct consistent with  $S_nAr$  mechanism by mass spectrometry. *Drosophila melanogaster* HhC with added ST044643 was separated by reverse phase HPLC and characterized by ESI-MS. Calculated mass of HhC (26247.8); calculated mass of HhC linked to butyl 3,5-dinitrobenzoate (26514.8).

*Materials:* Plasmids for arabinose-inducible expression of FRET-active hedgehog precursor protein, C-H-Y, and inactive control construct, C-H(Cys<sup>A</sup>Ala)-Y have been described previously (Owen et al., 2014). The FRET-active, point mutant of catalytic cysteine CysB, termed C-H(Cys<sup>B</sup>Asp)-Y, was prepared by standard cloning methods. Chemicals obtained commercially included: Bis-Tris Propane, NaCl, imidazole, glycerol, sodium phosphate, and Lennox Broth (LB) Mix (Fisher Chemical); cholesterol, 5-cholestene, arabinose, glucose, and Triton X-100 (Sigma Corp.); 25-NBD cholesterol (Avanti Polar Lipids); TCEP (tris(2-carboxyethyl)phosphine) (Hampton Research Inc.), Acti-Targ Protease Library (TimTec Inc.). Multi-well, NBS-coated black plates used in FRET assays were purchased from Corning Inc.

*Protein expression and purification:* FRET-active proteins, C-H-Y, C-H(Cys<sup>A</sup>Ala)-Y, C-H(Cys<sup>B</sup>Asp)-Y, and C-Y [1], each containing a C-terminal His<sub>6</sub> sequence were purified from *E. coli* strain LMG194 using Ni-NTA agarose, as described earlier [2]. Briefly, starter cultures (5 ml) from overnight growth in LB plus chloramphenicol (100 µg/ml) and glucose (0.2%), were transferred to 500 ml of LB with chloramphenicol (100 µg/ml), grown with shaking at 37 °C to an OD<sub>600</sub> of 0.6, then induced with arabinose (0.2% final). Following overnight incubation at 16 °C, cultures were pelleted and frozen at -80 °C. Thawed pellets were lysed by vortexing for 3-5 min in 15 ml of chilled phosphate buffer (pH 7.6) containing triton X-100 (0.5 %), glycerol (10%), potassium chloride (0.1 M), sodium chloride (0.1 M), lysozyme (10 µg/ml), DNAase (2 µg/ml) and imidazole (0.04 M). Purification using Ni-NTA spin column (ThermoFisher) was carried out according to manufactures protocol. C-H-Y remained active for months when stored at -80 °C in elution buffer with added glycerol (10%) and TCEP (4 mM). A typical elution would yield 5-7 mls of protein (20-30 µM) with >80% purity, sufficient for ~ 6000 wells using the 100 µl assays format described below.

*Cholesterolysis Microplate Assay:* Activity of C-H-Y was routinely measured at 30 °C using a BioTek H1 plate reader. Samples (100 µl, total) contained C-H-Y (0.2 µM) in Bis-Tris buffer (0.05 M, pH 7.1), EDTA (0.005 M), NaCl (0.1 M), TCEP (0.002 M) and Triton X-100 (0.4%). For simplicity the assay buffer is referred to as BENTT. After pre-incubating samples for 10 min at 30 °C, reactions were initiated with cholesterol from a stock in ethanol (0.025 M). In the assay, cholesterol concentration ranged from 50 to 250 µM; the final concentration of ethanol in the assay did not exceed 5% (v/v). Cholesterolysis of C-H-Y was monitored continuously by the decrease in FRET, where FRET = em 540 nm/em 460 nm following excitation at 400 nm.

*General Screening Procedure:* Experiments aimed at identifying cholesterolysis inhibitors are reported below in the format recommended by Inglese et al [3]

Event	Description	Parameter	Comments
Dispense	C-H-Y (0.2 μM) in BENTT buffer added to each well	96 μl	Solution may be frozen at -80 C without appreciable loss of activity
Dispense	Compounds added to a final concentration of 100 μM from DMSO stock	2 μl	DMSO added to intraplate controls in columns 1 and 12
Incubate / Read	Ex 400 nm, with Em 460 nm and 540 nm; wavelength switching; gain 100	30 °C, 20 min	Biotek Synergy 2 Plate reader; wells read at minimum time interval
Dispense	of cholesterol in ethanol stock to a final concentration of 60 μM	2 μl	Warm cholesterol solution to ensure dispersion
Read	Ex 400 nm, with Em 460 nm and 540 nm; wavelength switching; gain 100	30 °C, 120 min	Biotek Synergy 2 Plate reader; wells read at minimum time interval

#### *Secondary Screen:*

Secondary Screening of hits from TimTec library was carried out using a modified procedure previously described (Owen et al., 2014). The activity of the Hh precursor, SHhN-DHhC, was assayed in BENTT buffer. Hh precursor (4 μM, final) was titrated with ST044643 and allowed to incubate before initiating the reaction with 250 μM cholesterol. The cholesterolysis reactions were allowed to incubate at room temperature with aliquots taken at three and 24 hours. The reaction aliquots were quenched with SDS-PAGE loading buffer followed by boiling. Following separation by 15% SDS-PAGE and Coomassie blue staining, the extent of reaction was determined by the intensity of product bands (SHhN and DHhC) relative to precursor at each given [ST044643]. Gels were imaged and analyzed using Gel Doc™ EZ imager (BioRad) equipped with Image Lab Software. Cholesterolysis activity was determined by dividing the extent of processing at a given [ST044643] by the extent of processing in the absence of ST044643. An apparent IC<sub>50</sub> was determined by fitting the fractional activity as a function of [ST044643] to a dose-response equation given below:

$$Activity_{calc} = max + \frac{(min - max) * [ST044643]}{IC_{50} + [ST044643]}$$

1. Amitai, G., et al., *Modulation of intein activity by its neighboring extein substrates*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(27): p. 11005-10.
2. Owen, T.S., et al., *Active site targeting of hedgehog precursor protein with phenylarsine oxide*. Chembiochem : a European journal of chemical biology, 2014.
3. Inglese, J., C.E. Shamu, and R.K. Guy, *Reporting data from high-throughput screening of small-molecule libraries*. Nature chemical biology, 2007. **3**(8): p. 438-41.