Supplemental Information

Flotillin-1 is essential for PKC-triggered endocytosis and membrane microdomain localization of DAT

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Statistical analysis

Complete statistical analyses for each of the figures are provided below.

Fig.2b. ANOVA revealed a significant difference between Group (Ctrl vs Flot1 overexpression) ($p = 0.0078$), Dose of Go6850 ($p < 0.0001$) and an interaction between Group and Dose of Go6850 ($p = 0.0058$). Fisher PLSD post hoc comparison revealed a significant difference at 0.1 μM Go6850 ($p = 0.0231$).

Fig.3b. ANOVA revealed a significant difference between Group (siCtrl vs siFlot1) ($p < 0.0001$), Treatment (+PMA vs –PMA) ($p < 0.0001$) and an interaction between Group and Treatment ($p < 0.0001$). Fisher PLSD post hoc comparison revealed a significantly different effect of PMA treatment in siCtrl cells ($p < 0.0001$) but not in siFlot1 cells ($p = 0.226$).

Fig.3d. ANOVA revealed a significant difference between Group (siCtrl vs siFlot1) ($p = 0.0005$), and an interaction between Group and Treatment ($p = 0.0006$). Fisher PLSD post hoc comparison revealed a significantly different effect of PMA treatment in siCtrl cells ($p = 0.0003$) but not in siFlot1 cells ($p = 0.1038$).

Fig.4d. ANOVA revealed a significant difference between Group (RFP Ctrl vs Flot1 vs C34A) ($p < 0.0001$), Go6850 treatment ($p < 0.0005$) and an interaction between Group and Go6850 Treatment ($p = 0.0017$). Fisher PLSD post hoc comparison revealed a significantly different effect of Go6850 treatment on PMA-induced internalization between Ctrl and Flot1 cells ($p = 0.0021$) but not between Ctrl and C34A cells ($p = 0.1022$). In the absence of Go6850 treatment, no significant difference was seen between Ctrl and Flot1 cells ($p = 0.2415$) while there was a significant difference between Ctrl and C34A ($p < 0.0001$).

Fig.4f. ANOVA revealed a significant difference between Group (Flot1 vs C34A) ($p < 0.0001$), Treatment (+PMA vs –PMA) ($p < 0.0211$) and an interaction between Group and Treatment ($p = 0.0017$). Fisher PLSD post hoc comparison revealed a significant difference effect of PMA treatment in Flot1 cells ($p = 0.0148$) but not in C34A cells ($p = 0.1022$). A significant difference was also revealed between Flot1 and C34A cells in the presence ($p < 0.0001$) and absence ($p = 0.0369$) of PMA.

Fig.6b. ANOVA revealed a significant difference between Flotillin (Flot1 vs S315A) ($p < 0.0001$) and Treatment (Vehicle vs PMA vs PMA+Go (Go)) ($p < 0.0001$) and an interaction between Flotillin and Treatment ($p = 0.0224$). Fisher PLSD post hoc comparison revealed a significant difference between all comparisons within Flot1 (Ctrl vs PMA, $p = 0.0474$; Ctrl vs Go, $p = 0.0047$; PMA vs Go, $p = 0.0017$). In contrast, comparisons within Flot1(S315A) mutants, significant differences were revealed between Ctrl vs Go ($p < 0.0001$) and PMA vs Go ($p < 0.0001$), but not between Ctrl and PMA ($p = 0.1610$).

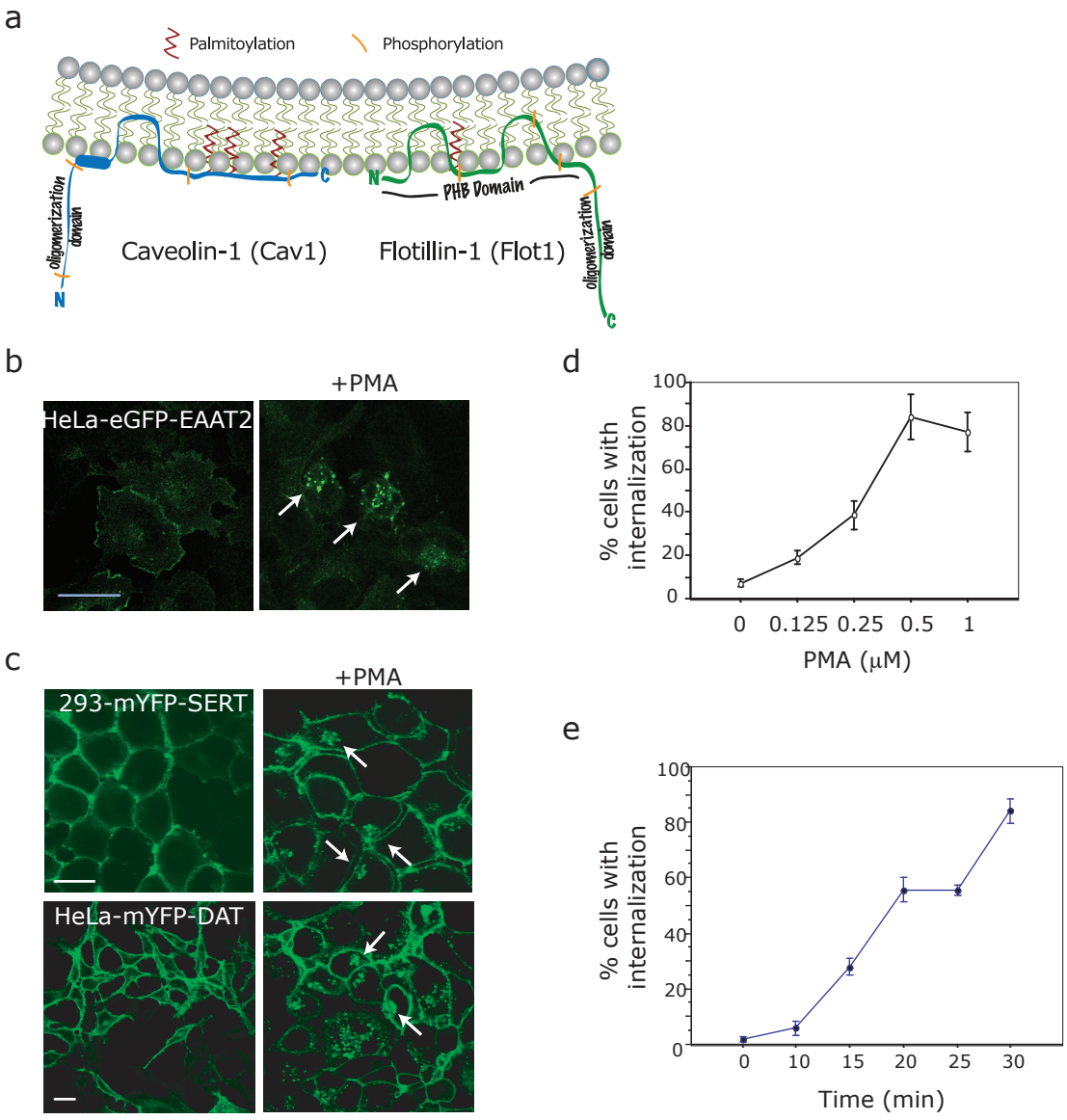
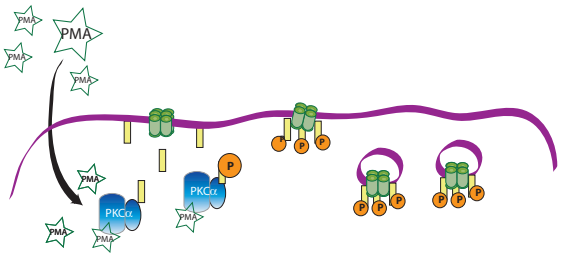
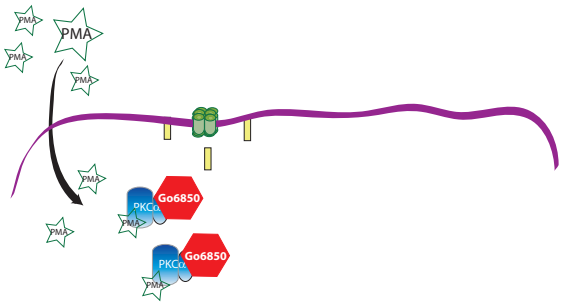


Fig. S1. PKC-triggered internalization of EAAT2, DAT and SERT in stably transfected cell lines.
a. Schematic representation of two clathrin independent endocytic proteins, Cav1 and Flot1.
b,cThe administration of 0.5 μM PMA leads to internalization of EAAT2 in HeLa-eGFP-EAAT2 cells (b) or HeLa-mYFP-tagged DAT (c) . Administration of 1 μM PMA leads to internalization of mYFP-tagged serotonin transporter in HEK293 cells (293-mYFP-SERT) as well.
d. Internalization occurs in a PMA dose-dependent manner as shown for EM4-YFP-DAT.
e. Time course of internalization of EM4-YFP-DAT after administration of 1 μM PMA. n = 100 cells per time point. Scale bar = 10μm.

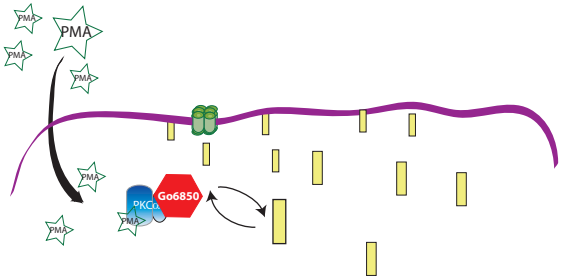
1. PKC phosphorylates substrate causing NTT internalization



2. Go6850 inhibits internalization by reversibly binding to the substrate binding site, and prohibiting its phosphorylation



3. Overexpression of substrate can outcompete the inhibitor for PKC



4. Phosphorylation leads to DAT internalization despite the presence of inhibitor

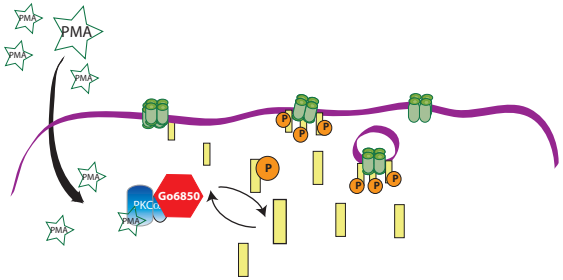


Fig. S2. Schematic representation of assay design used to identify PKC substrates. Go6850 (also known as Bisindolmaleimide I or GF 109203X) is a selective competitive inhibitor of PKC. Among the PKC isoforms, it is most selective for PKC α and β (IC50 = 10 - 20 nM), less selective for δ and ε (120 - 300 nM) and least selective for ζ (6000 nM).

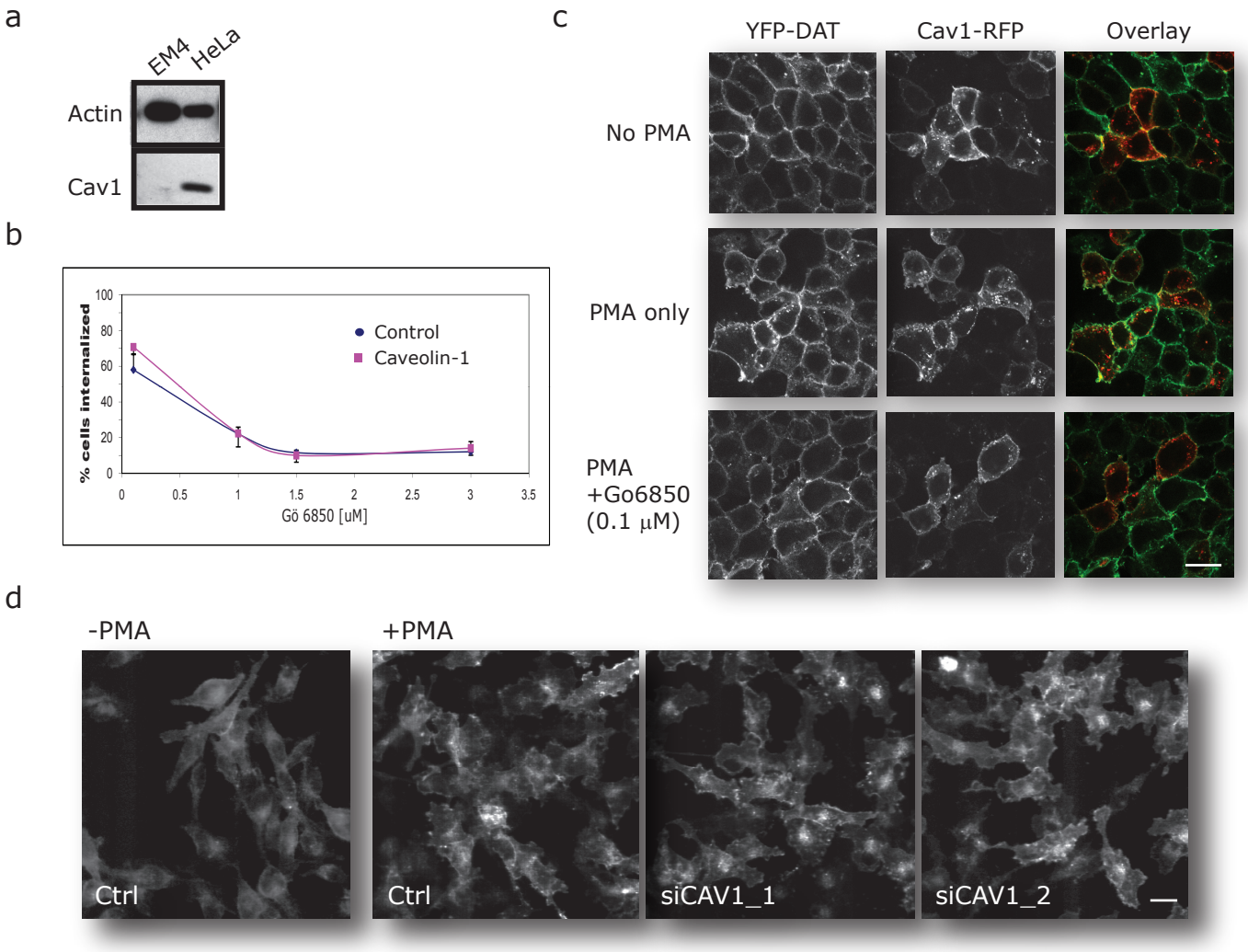
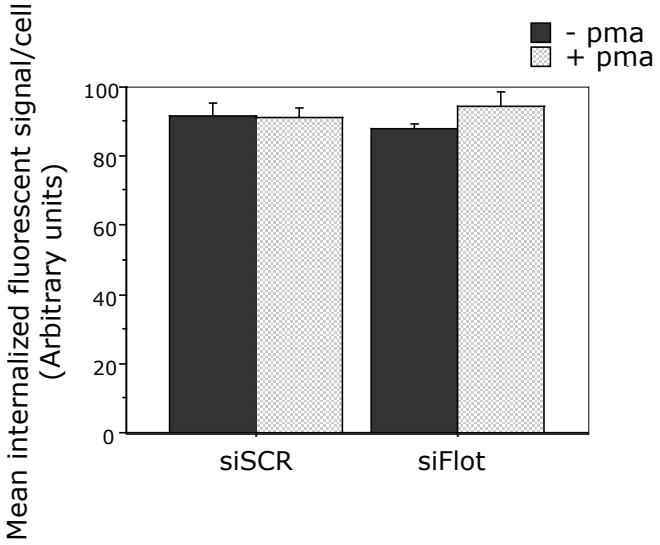


Fig. S3. Cav1 is not involved in PKC-regulated endocytosis a. Immunoblotting reveals that the HEK293-derived EM4 stable cell lines have little to no detectable levels of Cav1, as previously reported (see main text for references). b-c. Overexpression of Cav1 fails to overcome the Gö6850-mediated inhibition of PKC-triggered internalization of DAT in EM4-YFP-DAT Interestingly, despite over-expression of Cav1, DAT internalizes into compartments distinct from Cav1 in response to PMA, (1 μM, 30 min). D. Knockdown of Cav1 in HeLa-eGFP-DAT using 2 different siRNA against Cav1 (siCAV1_1, siCAV1_2) did not impede PKC-triggered endocytosis. Scale bar = 10 μm

a.



b.

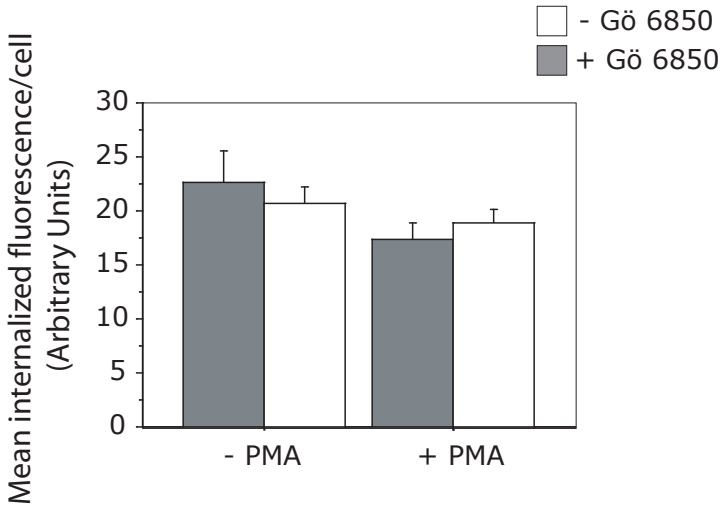


Fig. S4. a. Knockdown of Flot1 does not impede transferrin receptor (TfR) internalization. b. TfR internalization is insensitive to PKC activation by PMA or PKC inhibition by Gö 6850. For both experiments, EM4-YFP-DAT cell lines were exposed to Alexa Flour 633 labeled transferrin, incubated for 30 min at 37 degreesC and monitored for TfR internalization. Cells were measured for mean internalized fluorescence. Data is presented as mean ± SEM.

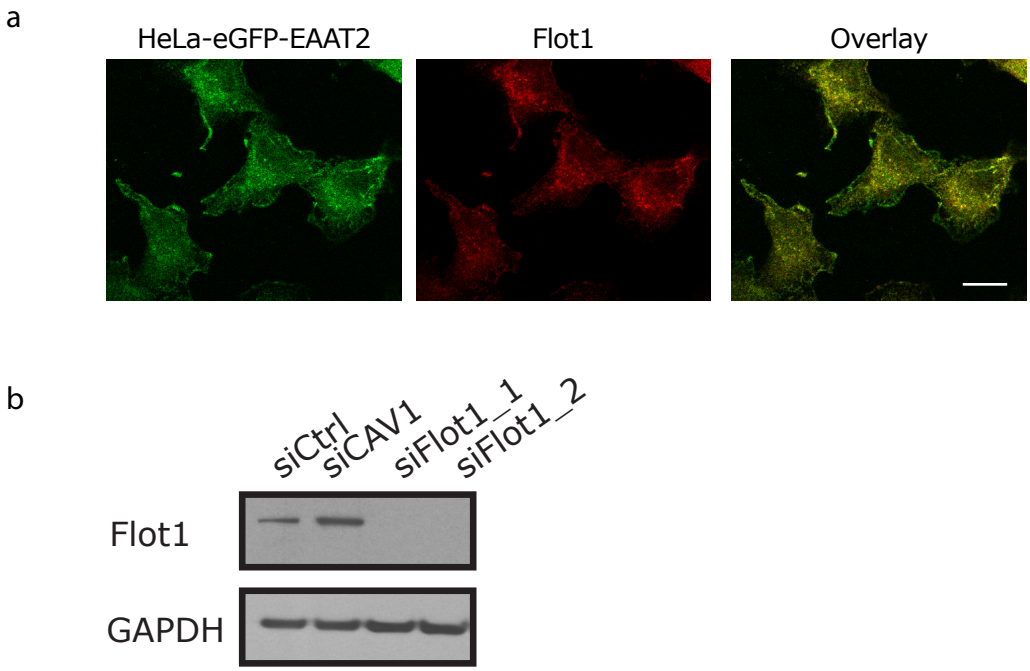


Fig. S5. EAAT2 internalizes into Flot1 positive vesicles after exposure to PMA. a. Hela-eGFP-EAAT2 stable cells were exposed to 0.5 μM PMA and fixed with methanol as described in Methods. Cells were probed with anti-Flot1 antibody (Santa Cruz Biotechnology), revealed with AlexaFluor 546 secondary antibody and imaged by confocal microscopy. Scale bar = 10 μm. b. siFlot1 efficiently knocks down Flot1 protein levels. Immunoblots of whole cell lysates of EM4-YFP-DAT cells transfected with one of two different siFlot1 (siFlot1_1 or siFlot1_2), control siRNA (siCTRL) or siRNA against Cav1 (siCav1).

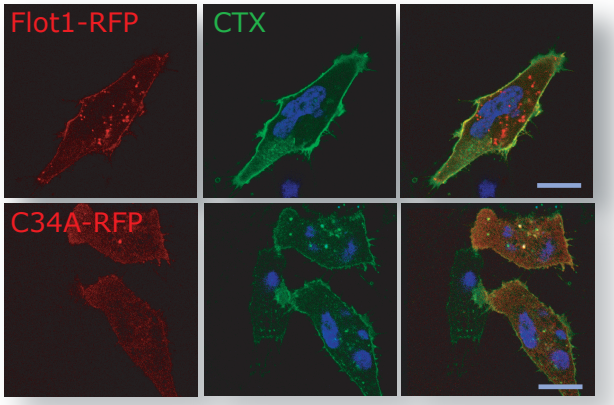
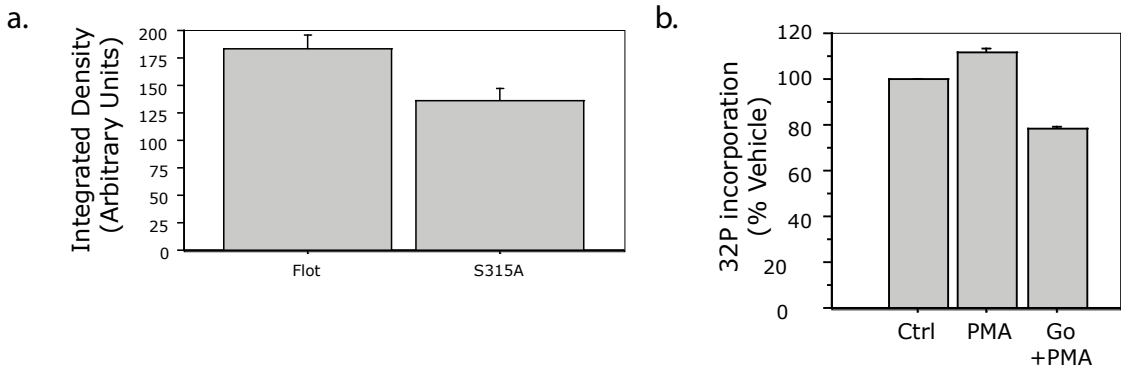


Fig. S6. Mutagenesis of Cys34 of Flot1 to Ala (Flot1(C34A)) changes the cellular localization of Flot1-RFP from membranous to largely cytosolic. Membrane staining reveals that although there is dramatically less C34A-RFP at the plasma membrane due to the mutation, it still retains some membrane localization (upper *versus* lower right-most panels). Wild type HeLa cells were transiently transfected with Flot1-RFP or Flot1(C34A)-RFP as described in Methods. Transfected cells were incubated with Cy3-tagged Cholera toxin B (CTX) subunit to stain for membrane (Molecular Probes), and imaged by confocal microscopy. Scale bar = 10 μm.

	K_M (μM)	V_{MAX} (%)
Vehicle	12.08+0.08	105.0+6.00
MbC 20	5.70+0.24	36+3.80
MbC 60	7.20+0.05	1.50+0.13

Table S1. MbC inhibits DAT-mediated uptake of tyramine in a dose-dependent manner. EM4-YFP-DAT cells are either serum deprived for 1 h and administered vehicle, 20 mM or 60 mM MbC. Cells are then assayed in a [^{3}H]-tyramine uptake assay. Km and Vmax are calculated as previously described[5]. Data presented as Mean+St.Dev (n=4).



FigS7. Further quantification to accompany the metabolic labeling experiment of Flot1 in response to PKC activation (Fig.6b). a. Flot1(S315A) phosphorylation mutant overall demonstrated significantly less ^{32}P-orthophosphate labeling than wildtype Flot1. Bars represent Mean+SEM of integrated densities in arbitrary units (AU) (ANOVA, *p=0.0146, n=2). b. PMA administration leads to a significant increase of ^{32}P labeling of Flot1 indicating PKC activated phosphorylation of Flot1 (ANOVA, *p=0.0098, n=4). Preincubation with Go6850 significantly blunts this effect suggesting that not only it inhibits PMA-induced phosphorylation, but may also decrease some baseline phosphorylation of Flot1. ^{32}P-orthophosphate incorporation represented as % Vehicle.

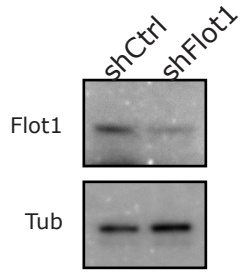


Fig S8. shRNA against Flot1 (shFlot1) versus a nonsilencing shRNA control vector. Primary neurons were transduced with lentivirus at DIV3, and lysates were collected 72 hrs later. Loading control is Tubulin (Tub).

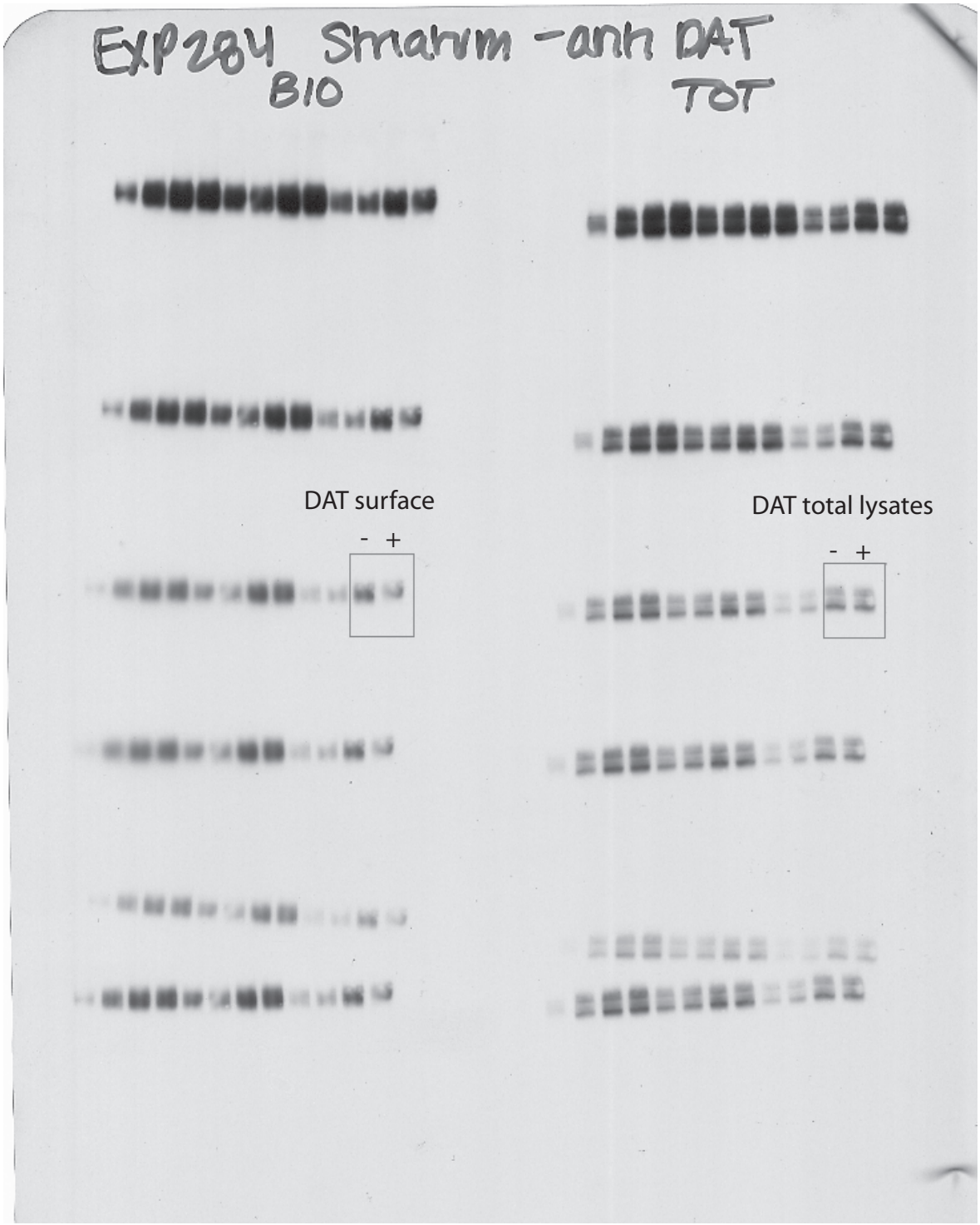


Fig. S9. Western blots for Figure 1.

Fig.2A

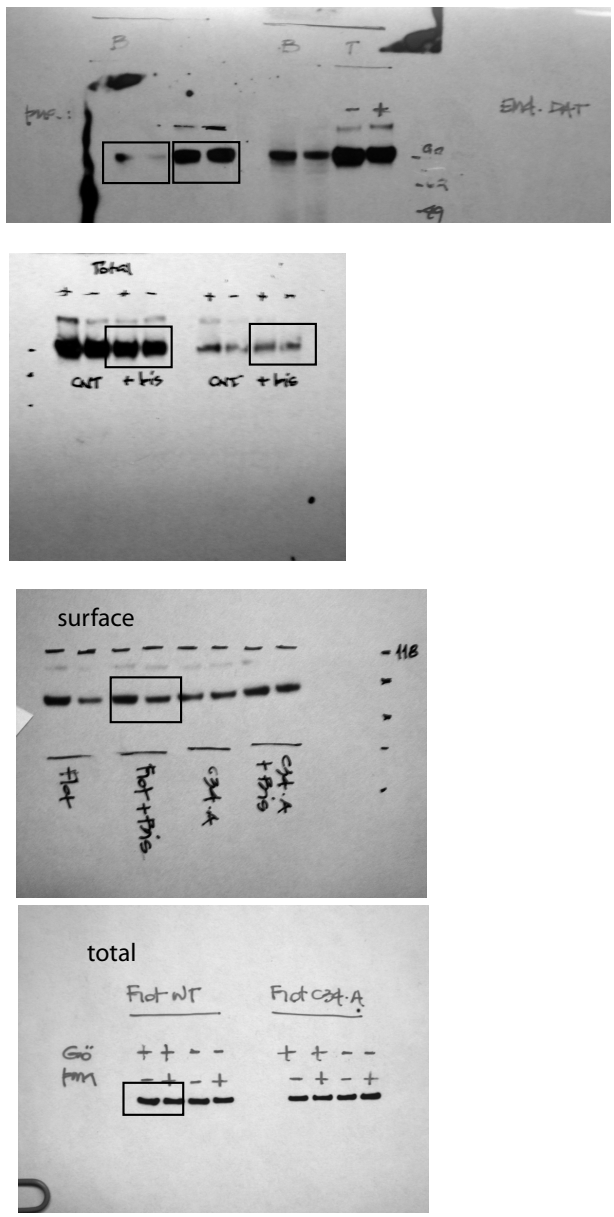


Fig. S10. Western blots for Figure 2a.

Fig. 3a

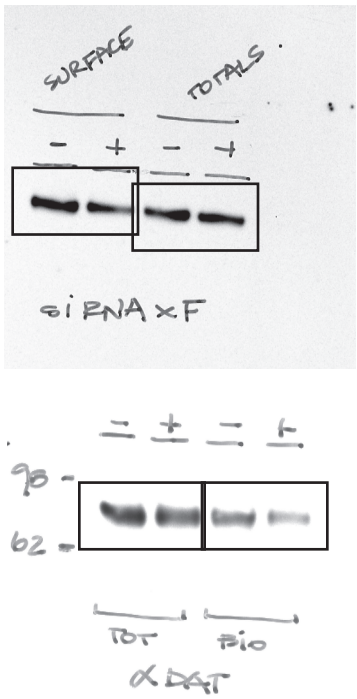


Fig 3d

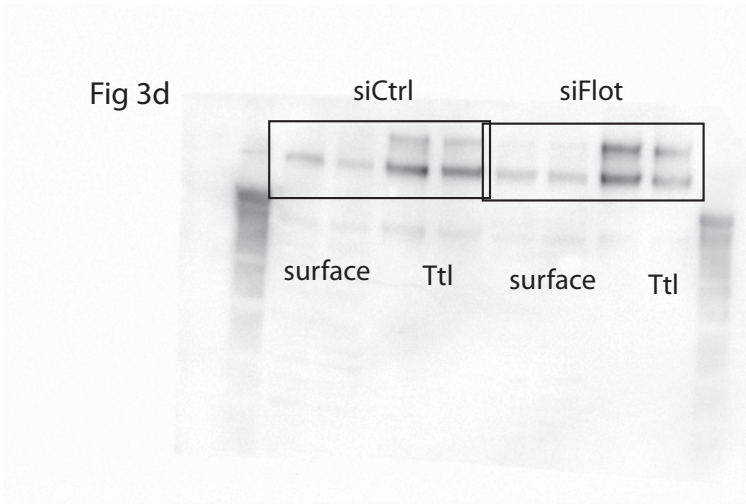
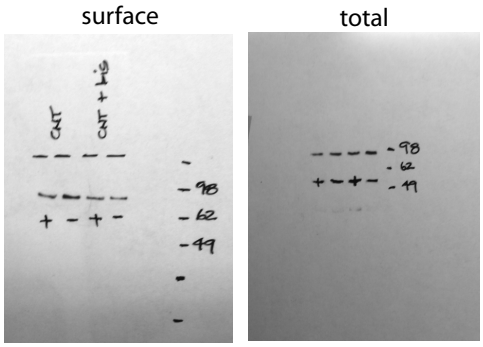
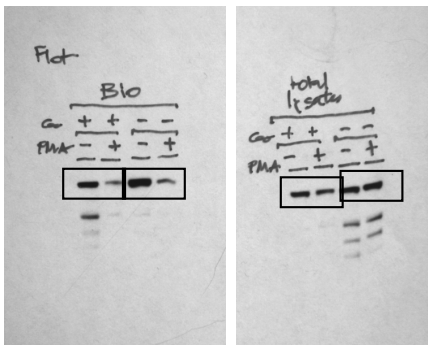


Fig S11. Western blots for Figure 3

Fig 4c





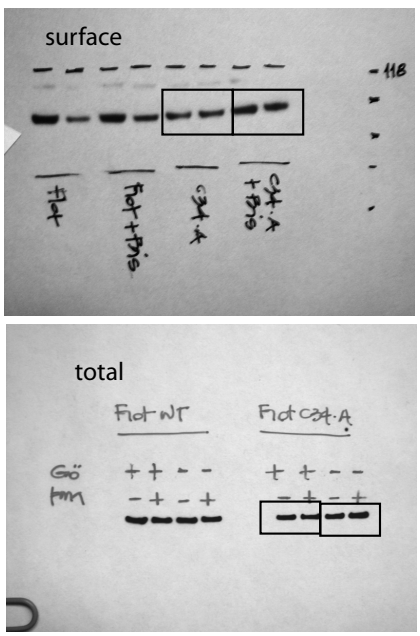


Fig 4e

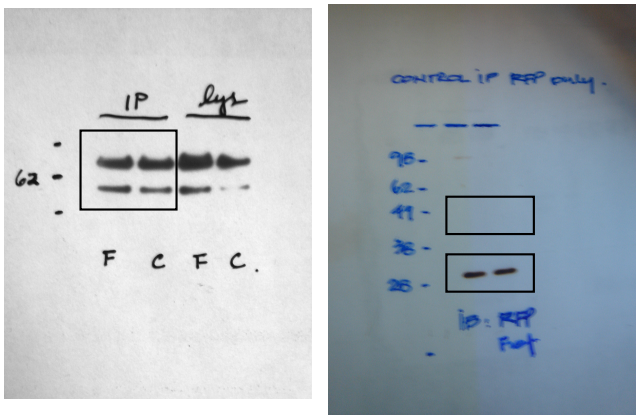
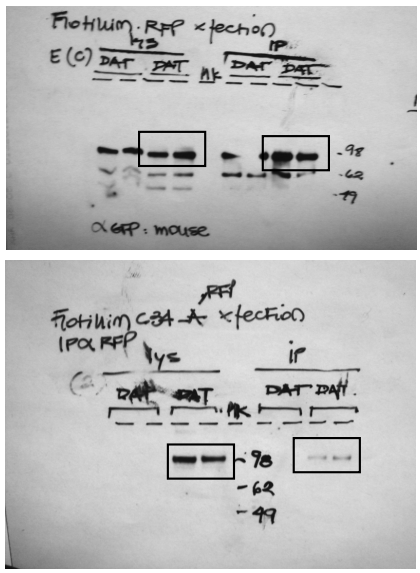
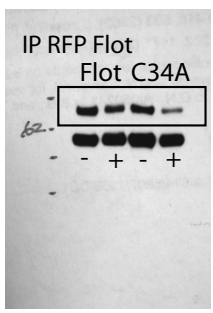


Fig 4f





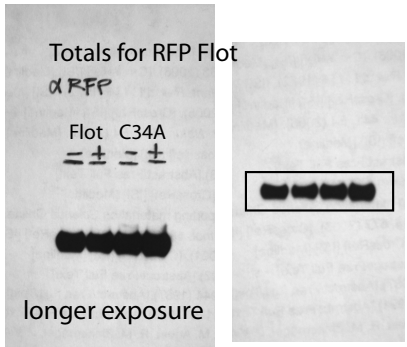


Fig. S12. Western blots for Figure 4

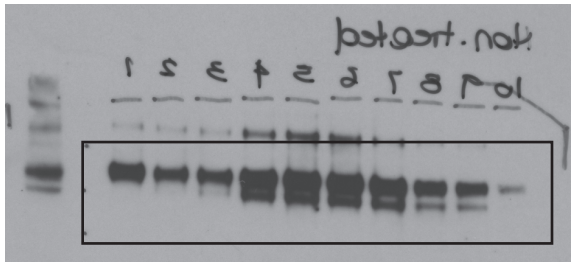
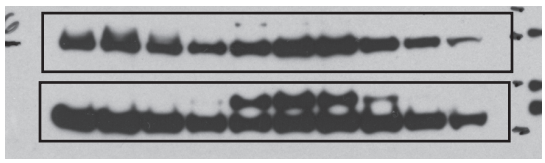


Fig. 5a



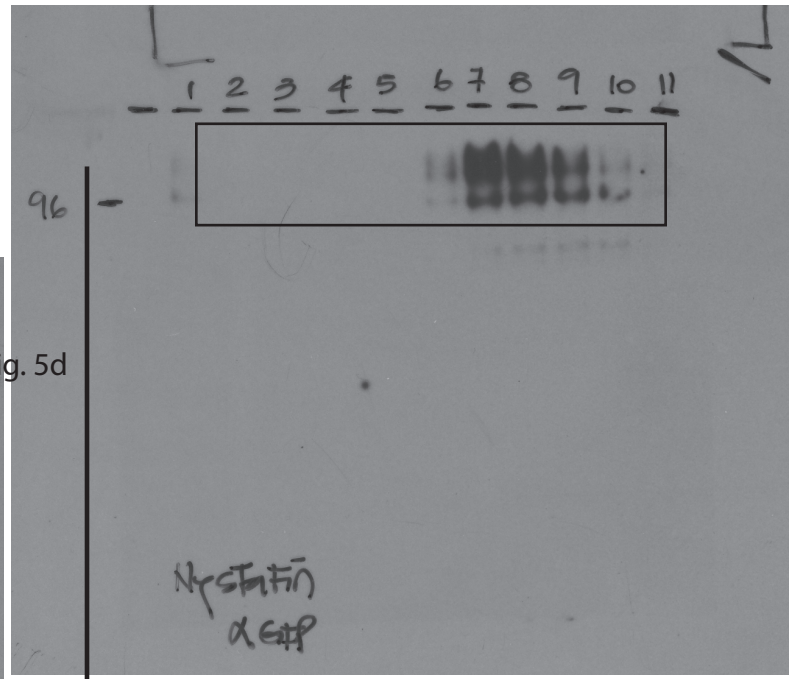


Fig. 5d

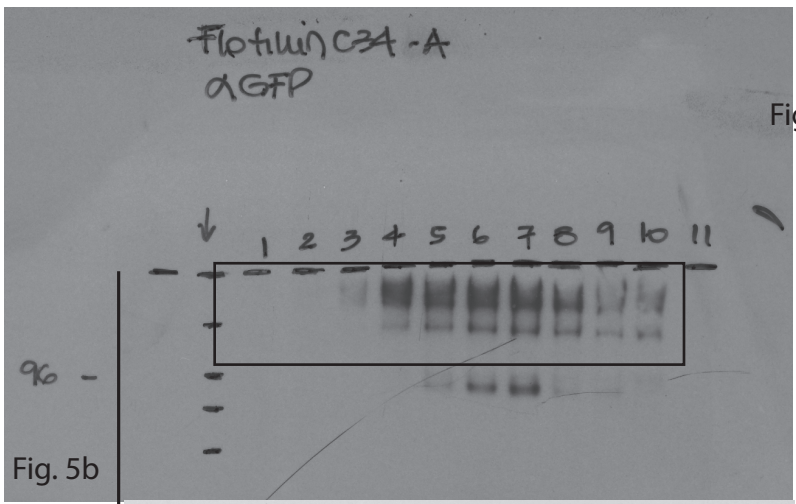
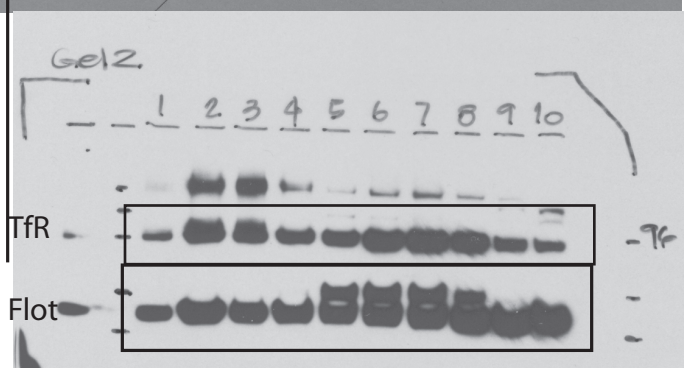
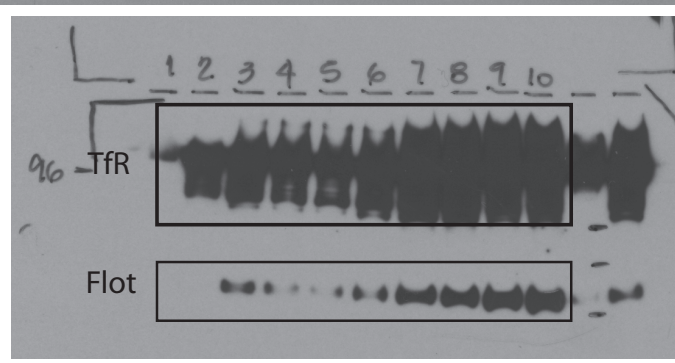
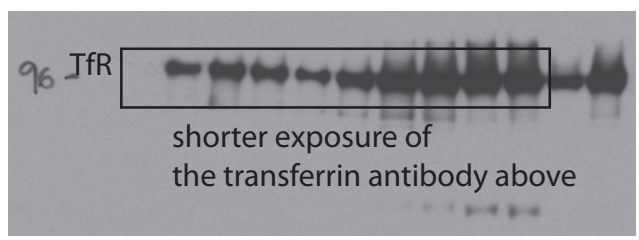


Fig. 5b







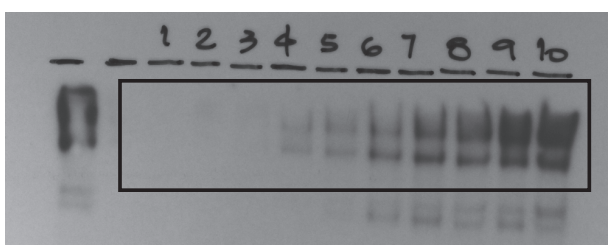


Fig. 5c

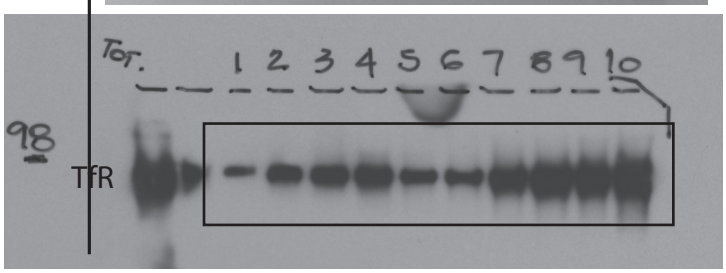
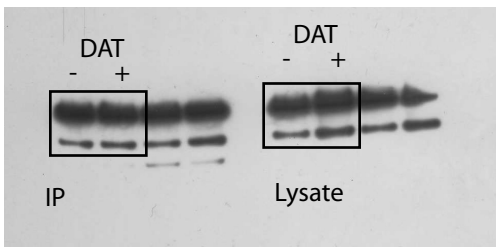
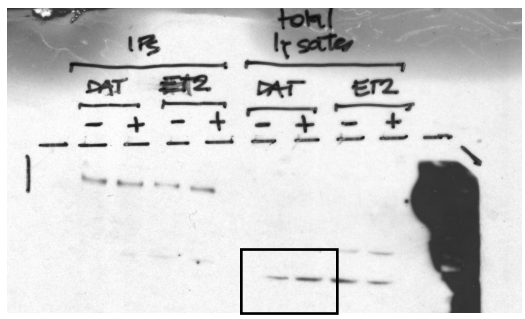


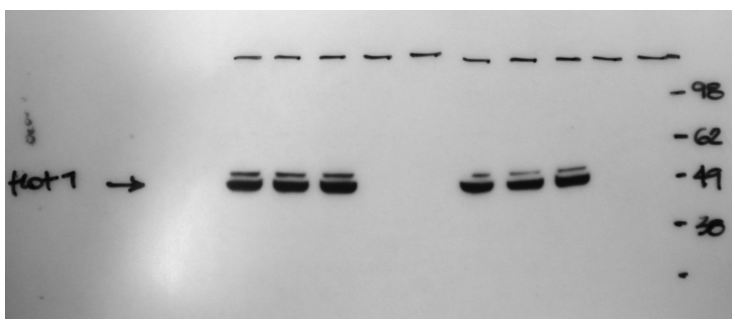
Fig. S13. Western blots for Figure 5

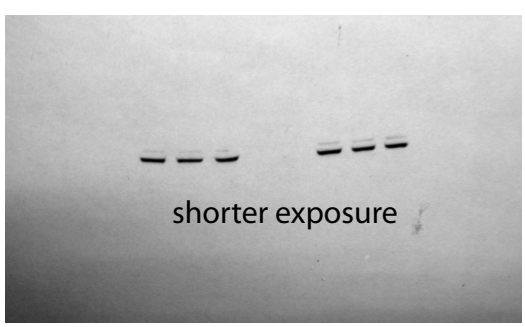
6a





6b





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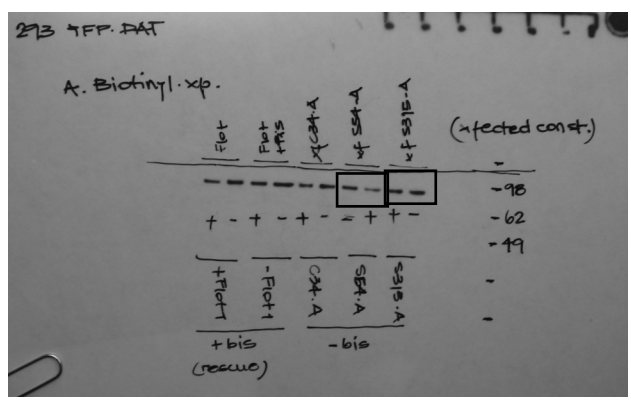

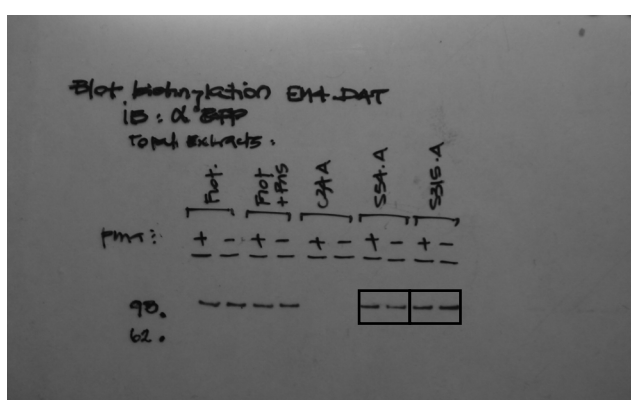

Fig. S14. Westerns for Figure 6.