Supplemental Materials
Molecular Biology of the Cell

Luscher et al.

Supplementary Figure 1, related to Figure 1. Determination of candidate F&H proteins in humans by peptide array. **A**) Western Blot on three candidate proteins in HKC lysates showed no specific interaction with the OCRL F&H interface. **B**) ITC reveals the putative F&H peptide of Dynein heavy chain to be a non-specific interactor. **C**) Summary of tested human F&H peptides. Proteins labelled in red have been validated in previous publications. Proteins in yellow have been confirmed to be non-interactors of the OCRL-F&H motif by Isothermal Titration Calorimetry. Red residues are common to Ses1/2 and APPL1, blue residues are conserved in Ses proteins. Purple residues are at key positions and fall outside the Ses/APPL1 consensus. (MS= mass spectrometry on GFP-OCRL precipitates), WB (Western Blot) ITC (Isothermal titration calorimetry). + published in Swan et al PNAS 2010 and Pirruccello et al NSMB 2011; * Nandez et al eLIFE 2014.

Supplementary Figure 2, related to Figure 2. Sequence conservation between Dd5P4 and human INPP5B and OCRL. Domains are indicated by lines under the sequence alignment. *Identical residues* are shaded. *Boxed residues*: interaction motifs for clathrin and AP2 (unique to OCRL). *Dot*: catalytic residue. *Arrowhead:* key Rab binding residue. *Starred residues*: mediate contact with F&H peptides.*Residues marked in red:* mutated in this study.

Supplementary Figure 3, related to Figure 2: Analysis of peptide enrichment of GFP immunoprecipitates from **A**) WT vs *Dd5P4*⁻;GFP-Dd5P4^{WT} and **B**) WT vs *Dd5P4*⁻;GFP-Dd5P4^{W620A} shows that putative F&H proteins (**Figure 2B**) are specifically enriched in precipitates from GFP-Dd5P4^{WT} expressing cells.

Supplementary Figure 4: related to **Figures 3 and 4**. Sequences of PIO and GxcU proteins **A)** The predicted PH domains of *D discoideum* PIO (aa 543-741) and *D. purpureum* PIO partial sequence (aa 200-401). Identical residues are shaded. *Boxed region* was used to search crystal structures with Phyre2 **B)** Conservation of amino acid sequence between *D discoideum* and *D intermedium* GxcU genes. The Rho-GEF, PH and FYVE domains are strongly conserved, as is the F&H peptide sequence (boxed residues).

Supplementary Figure 5, related to Figure 6: GFP-Dd5P4^{W620A} rescues many aspects of *Dd5P4$^-$* dysfunction. **A)** N-terminal and C-terminal GFP fusions of Dd5P4, and an N-terminal GFP labelled mutant of the F&H surface (W620A) all restore growth in a *Dd5P4$^-$* background. N=3 independent growth curves per genotype. **B)** Both GFP-Dd5P4 and GFP-Dd5P4^{W620A} are largely cytosolic (as seen in previous publications), but GFP fluorescence is found on cytosolic organelles which are not part of the endocytic system (negative for 7KDa Dextran label).Arrowheads show GFP accumulation at collapsing contractile vacuole. **C)** Re-expression of GFP-tagged Dd5P4 restores osmotically-triggered CV fusion in cells labelled with FM4-64, N=3 independent movies per genotype, normalised to corresponding wildtype value. **D)** Lifetime of GFP-labelled CVs imaged at 0.2Hz *prior* to fusion in *Dd5P4$^-$*; GFP-Dd5P4^{WT} cells is not significantly different to that of *Dd5P4$^-$*; GFP-Dd5P4^{W620A} expressers: n= 28 GFP-Dd5P4^{WT} labelled events, n=67 GFP-Dd5P4^{W620A} labelled events, Student's t-test. All CV exocytosis events which were GFP positive either in the pre-collapse or collapse phase were analysed. The majority of GFP-Dd5P4^{WT} CVs are positive for GFP only in the collapse phase (i.e are recorded as zero frames before CV collapse).

Supplementary Movie 1, related to Figure 1: Ruby-OCRL (red) and Frabin-GFP (green) are found together on pinosomal compartments in Cos7. Images 0.25Hz. Scale Bar 3μm.

Supplementary Movie 2, related to Figure 4: GxcU-GFP can be detected on phagosomes in WT cells. Images 0.25Hz. Scale Bar 15μm.

Supplementary Movie 3, related to Figure 4: GxcU-GFP can be detected on phagosomes in *Dd5P4*⁻ cells. Images 0.25Hz . Scale Bar 15μm.

Supplementary Movie 4, related to Figure 5: Recruitment of a C-terminal GFP fusion of OIBP (green). Contractile vacuoles are labelled by FM4-64 (red). Images 0.25Hz. Scale Bar 10μm.

Supplementary Movie 5, related to Figure 6: 1:1 dilution of imaging medium with distilled water caused an increase CV exocytosis rate in WT. Images 0.2 Hz. Scale Bar 15μm.

Supplementary Movie 6, related to Figure 6: 1:1 dilution of imaging medium with distilled water in *Dd5P4*⁻ mutants does not change CV exocytosis rate. Images 0.2 Hz. Scale Bar 15μm.

Supplementary Movie 7, related to Figure 6: the collapse/fusion phase of CV exocytosis in *Dd5P4*⁻ mutants re-expressing GFP-Dd5P4. Images 0.2Hz. Scale Bar 15μm.

Supplementary Movie 8, related to Figure 6: the collapse/fusion phase of CV exocytosis in *Dd5P4*⁻ mutants re-expressing F&H mutant GFP-Dd5P4^{W620A}. Images 0.2Hz. Scale Bar 15μm.
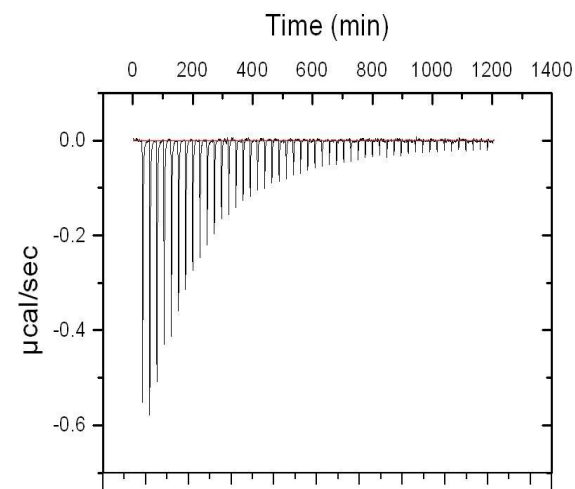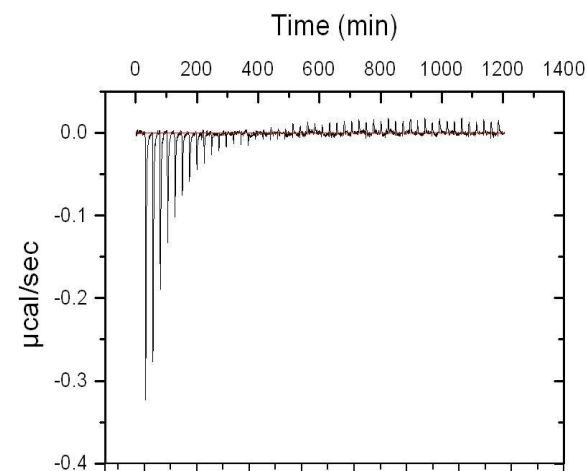
A

GST-Pulldown
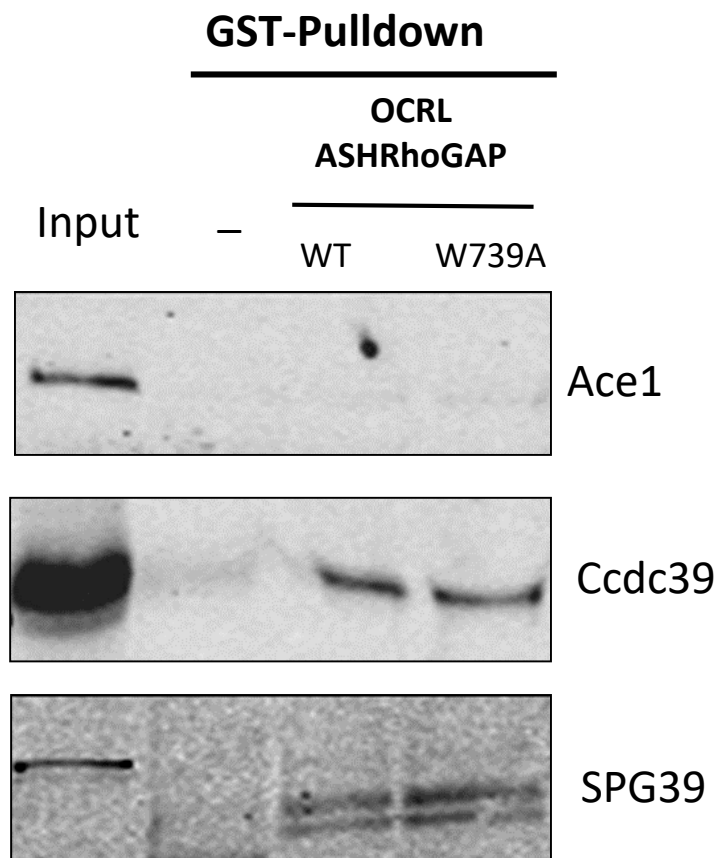
B

Isothermal Titration Calorimetry OCRL ASH-RhoGAP

Dynein heavy chain:
KFRRQHEQLRAVI

Dynein heavy chain mut.:
KARRQHEQLRAVI

C

Peptide	Protein	M.S$^{(*)}$	GST-PD	W.B	Array	ITC$^{(+)}$
Human proteins tested						
CFSTLHDWYGQEI	**hSes2**	**Y**	**Y**	**Y$^{(+)}$**	**Y**	–
PFARLHECYGQEI	**hSes1**	**Y**	**Y**	**Y$^{(+)}$**	**Y**	**Y$^{(+)}$**
SFQQRHESLYRP	**hAPPL1**	**N**	**Y**	**Y$^{(+)}$**	**Y**	**Y$^{(+)}$**
AFHQRHETFRNAI	**Frabin**	**N**	**Y**	**Y**	**Y**	–
IFHLYHKKTLFYS	FGD5	N	N	–	–	–
VFQLLHKNMLFYV	FGD6	N	N	–	–	–
DFIMMHCVFMPNT	SPG39	N	–	**N**	**Y**	–
LFSIRHRSLHRHS	Ace1	N	–	**N**	**Y**	–
DFRKIHNERQELI	Ccdc39	N	–	**N**	**Y**	–
VFSQLHELRQQDL	WDR81	N	–	–	**Y**	–
RFRKLHCTRNFIH	PACR	N	–	–	**Y**	–
KFRRQHEQLRAVI	Dynein.h.c	N	–	–	N	N
PFARLAECYGQEI	hSes1(mut)	–	N$^{(+)}$	N$^{(+)}$	N	N$^{(+)}$
Dictyostelium proteins tested						
DFSSQHQQYSFDQ	**OIBP**	**Y**	**Y**			
DFLTLHKAYIDEW	**PIO**	**Y**	**Y**			
SFIATHTAYNKEN	**GxcU** ($^{\#}$2)	**Y**	**Y**			
SFQLIHPIKSFTL	GxcU ($^{\#}$1)	Y	N			
GFVYIHCNLGLFK	Hwr ($^{\#}$1)	Y	N			
CFGTTHFCDTCHD	Hwr ($^{\#}$2)	Y	N			

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dd5p4     --------------MGDIQNTDNIESNIDNNNNNNVSLESS---SSSQ----------------------------NNNTN  36
hINPP5B   MDQSVAIQETLAEGEYCVIAVQGVLCEGDSRQSRLLGLVRYRLEHGGQEHALFLYTHRRMAITGDDVSLDQIVPVSRDFT  80
hOCRL     ------MEPPLPVGAQPLATVEGMEMKGPLREPCALTLAQR----NGQ-YELIIQLHE------KEQHVQDIIPINSHFR   63

dd5p4     -----------TTTTTTTSVDNLQVG------------------------------------------------------  52
hINPP5B   LEEVSPDGELYILGSDVTVQLTAELSLVFQLPFGSQTRMFLHEVARACPGFDSATRDPEFLWLSRYRCAELELEMPTPR   160
hOCRL     ---------CVQEAEETLIDIASNS-------GCKIRVQGDWIR--------------------ERRFEIPDEE       102

dd5p4     ----VLS-------ISDQSTPT----------------------------------------------------IET   66
hINPP5B   GCNSALVTWPGYATIGGGRYPSRKKRWGLEEARPQGAGSVLFWGGAMEKTGFRLMERAHGGGFVWGRSARDGRRDEELEE  240
hOCRL     HCLKFLS-----AVLAAQKAQS-------QLLVPEQKDSSSWYQKLDTKDKPSVFSGLLG---------------FED   153

dd5p4     PNQQQQQQQDDNNRGVSNETIKASLDG------LKHNSLKTTFP---------NST-------------------NHQY  111
hINPP5B   AGREMSAAAGSRERNTAGGSNFDGLRPNGKGVPMDQSSRGQDKPESLQPRQNKSKSEITDMVRSSTITVSDKAHILSMQK  320
hOCRL     NESMNLDKKINSQNQPTGIHREPPPP-----PFSVNKMLPREKEAS----NKEQPKVTNTMR--------KLFVPNTQS  216

dd5p4     IDVNTQWITNKLKERESEFTEKRGMSIFLGTWNVNGKKPSESLDPWLKDPSMSLQPDIYAIGFQELDLTAEALLLGDTTR  191
hINPP5B   FGLRDTIVKSHLLQKEEDYTYIQNFRFFAGTYNVNGQSPKECLRLWLSNGIQ--APDVYCVGFQELDLSKEAFFFHDTPK  398
hOCRL     -GQREGLIKHILAKREKEYVNIQTFRFFVGTWNVNGQSPDSGLEPWLNCDPN--PPDIYCIGFQELDLSTEAFFYFESVK  293

dd5p4     SLPWEQHILNTLQG--DYVKLLSKQLVGILLCVYVKKEHKPHIANVQSDIAAVGIMGMMGNKGGVAIRFSFYNTTICILN  269
hINPP5B   EEEWFKAVSEGLHPDAKYAKVKLIRVGIMLLLYVKQEHAAYISEVEAETVGTGIMGRMGNKGGVAIRFQFHNTSICVVN   478
hOCRL     EQEWSMAVERGLHSKAKYKKVQLVRLVGMMLIFARKDQCRYIRDIATETVGTGIMGKMGNKGGVAVRFVFHNTTFCIVN   373

dd5p4     SHLNAHMDNVLRRNQDMKDISKNIKFINESSTDHSTINIFDHDQLFWIGDLNYRIPLPD-NEVKEKIKKKDFYNLFLVDQ  348
hINPP5B   SHLAAHIEEYERRNQDYKDICSRMQFCQPDPS-LPPLTISNHDVILWLGDLNYRIEELDVEKVKKLIEEKDFQMLYAYDQ  557
hOCRL     SHLAAHVEDFERRNQDYKDICARMSEVVPNQT-LPQLNIMKHEVVIWLGDLNYRLCMPDANEVKSLINKKDLQRLLKFDQ  452

dd5p4     LNQQMKAGAVFEGFQEPPISFAPTYKYDAGTEEYDSSEKKRTPAWCDRILWKTHKKAENVGILSYK-RAELISSDHRPVS  427
hINPP5B   LKIQVAAKTVFEGFTEGELTFQPTYKYDTGSDDWDTSEKCRAPAWCDRILWK----GKNITQLSYQSHMALKTSDHKPVS  633
hOCRL     LNIQRTQKKAFVDFNEGEIKFIPTYKYDSKTDRWDSSGKCRVPAWCDRILWR----GTNVNQLNYRSHMELKTSDHKPVS  528

dd5p4     ASFVIKIKVVIPDSKNRIYQEIVKELDKKENDSMPDANISTNMVDFETIKFMQPISKQLIFENIGQVIARFQFIPKLDET  507
hINPP5B   SVFDIGVRVVNDELYRKTLEEIVRSLDKMENANIPSVSLSKREFCFQNVKYMQLKVESFTIHN-GQVPCHFEFINKPDEE  712
hOCRL     ALFHIGVKVVDERRYBKVFEDSVRIMDRMENDFLPSLELSRBEEVFENVKFRQLQKEKFQISNNGQVPCHFSEIPKLNDS  608

dd5p4     ILCKPWLKISPLAGMMIPKEKVTIDLTIYVDNLTSGLFNINNNSTNSTNESMDDILILHLENGKDYFISISGKFQKTCFG  587
hINPP5B   SYCKQWLNANPSRGFLLPDSDVEIDLELFVNKMTATKL-------NSGEDKIEDILVLHLDRGKDYFLSVSGNYLPSCFG  785
hOCRL     QYCKPWLRAEPFEGYLFPNETVDISLDVYVSKDSVTIL-------NSGEDKIEDILVLHLDRGKDYELTISGNYLPSCFG  681

dd5p4     NTLDNLVRYPHPIRNN--------------------LPIPPEQKK------LSIPKELWRIIDYIYYNGLKEEGLFIKSG  641
hINPP5B   SPIHTLCYMREPILDLPLETISELT----------LMPVWTGDDGSQLDSPMEIPKELWMMVDYLYRNAVQQEDLFQQPG  855
hOCRL     TSLEALCRMKRPIREVPVTFLIDLEDSFLEKEKSLLQMVPLDEG-ASERPLQVPKEIWLLVDHLFKYACHQEDLFQTPG  760

dd5p4     VTKEMELIRDCLDTAEPFSSISFSIHSMAETLIRFLESLVEPVIPFNMYQQALDASSSPLSCKTLVSHLPSVNYNVFFYL  721
hINPP5B   LRSEFEHIRDCLDTGMIDN-LSASNHSVAEALLLFLESLPEPVICYSTYHNCLECSGNYTASKQVISTLPIFHKNVFHYL  934
hOCRL     MQEELQQIIDCLDTSIPET-IPGSNHSVAEALLIFLEALPEPVICYELYQRCLDSAYDPRICRQVISQLPRCHRNVFRYL  839

dd5p4     ISFLIETLSNQKENDLKPDQLAIIFSTVLLRPSPQSQLSQFPPDATTVKKKADLILHFLISKDLIN              787
hINPP5B   MAFLRELLKNSAKNHLDENILASIFGSLLLRNPAG----HQKLDMTEKKKAQEFIHQFLCNPL              993
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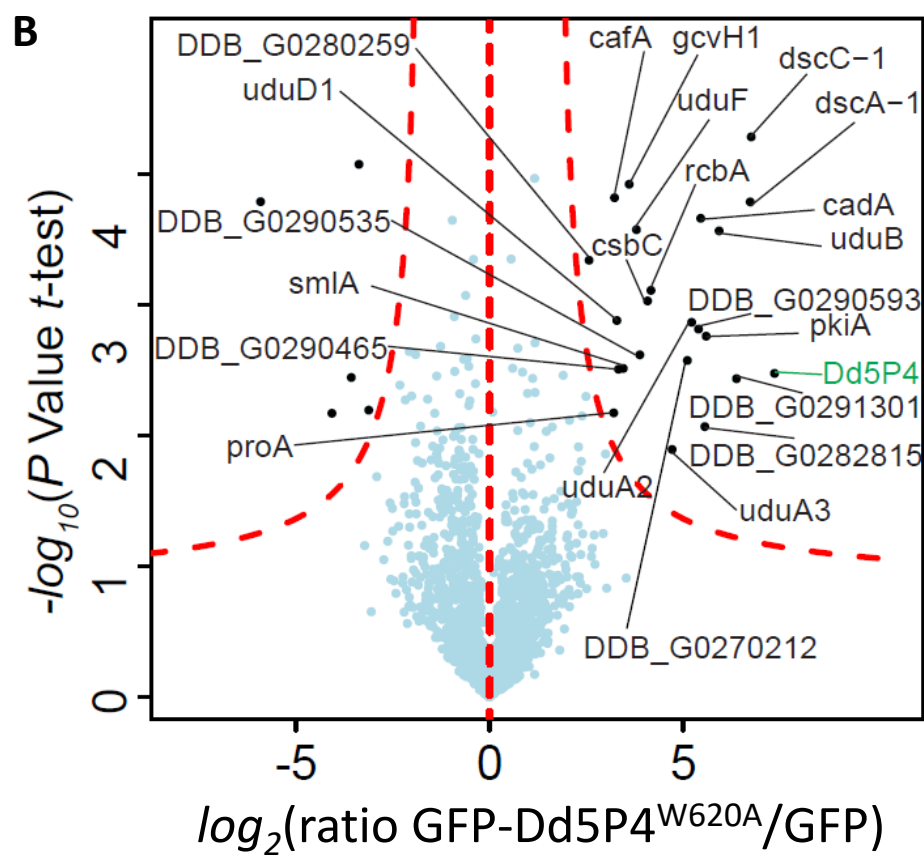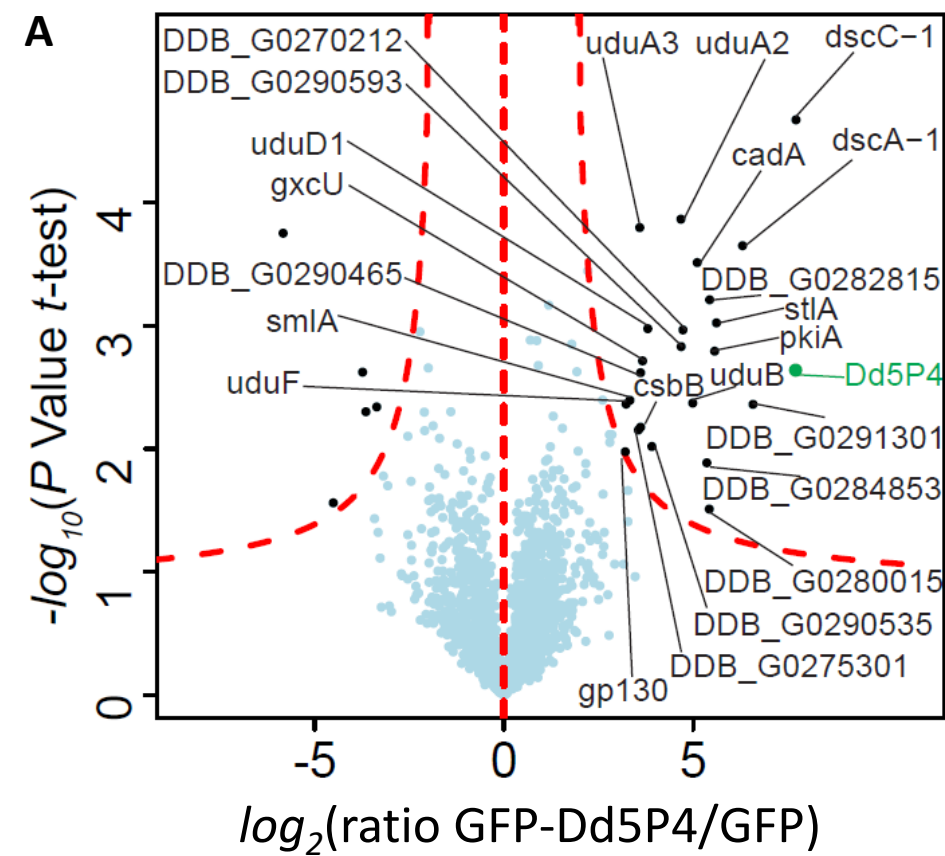
Legend: 5' Phosphatase   ASH   RhoGAP   Clathrin/AP2 binding   F&H surface

A

$-log_{10}(P\text{ Value } t\text{-test})$

$log_2(\text{ratio GFP-Dd5P4/GFP})$

DDB_G0270212
DDB_G0290593
uduD1
gxcU
DDB_G0290465
smlA
uduF
uduA3 uduA2 dscC-1
cadA dscA-1
DDB_G0282815
stlA
pkiA
csbB uduB Dd5P4
DDB_G0291301
DDB_G0284853
DDB_G0280015
DDB_G0290535
DDB_G0275301
gp130

B

$-log_{10}(P\text{ Value } t\text{-test})$

$log_2(\text{ratio GFP-Dd5P4}^{W620A}/\text{GFP})$

DDB_G0280259
uduD1
DDB_G0290535
smlA
DDB_G0290465
proA
cafA gcvH1
uduF dscC-1
dscA-1
rcbA
cadA
uduB
csbC
DDB_G0290593
pkiA
Dd5P4
DDB_G0291301
DDB_G0282815
uduA2
uduA3
DDB_G0270212

A

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D. dic PH  IIKQGWMKKRGTKNKSWKKRYFILDMKKTLRYYKDNSK--IPHGNNSNNGGGGSSSNNNNCNSGNNNNGLNGGSGKGDQV
D. pur PH  IIKQGWMKKRGTKNKSWKKRYFILDMKKTLRYYKDKSHHSVNNGNNGNNNNCNGNGKNDQIIVNNISSPINSNPHLSGST

D. dic PH  NGNNISLPIGNNPHFHSSSGSGIPTTSPSMSS-SSTSLENNDILSSFDHLKYKGSIDLYTSLVVAIKPSTFNVNINNSGS
D. pur PH  NNNININNPNNNNNNNINNSIN--NTNSISLSSNSSSSTSNLYENTSFENLRYKGSIDLYTSLVVAIKPNTCNVNINNNVN

D. dic PH  FKEDNNSYFGMDIITPSRTWNFCCESSKSMEDWLIVLKS--ANK
D. pur PH  IKEDNTQYFGMDIITPKRTWNLCCDSSKSMDEWLLALKSVQINK
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B

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D discoideum GxcU   MDTKNKEILGSTIKAWIGRKRYKTMMVNKTNRDKVAKEILDTEQIYVRNLETIVQ-FYLKPLRNINPPLLSPKSIQIIFGHIEDLLSLNTELLTSIQDRM  99
D intermedium GxcU  MDTKNKEILGSTIKAWIGRKRYKGMMVNKTNRDKVAKEILDTEQIYVRNLEFIVQVFYLKPLRNINPPLLSPKSIQIIFGHIEDLLSLNTELLISIQDRM  100

D discoideum GxcU   TTWYQDKRLGDIFTKMAPYLKVYTEYCGNYDKAVARLKQKSDESRDLQMFLKRVTSENSFGLDLNSLLVTPIQRIPRYKLLLQSLIQLTPVEFSDYPTLA  199
D intermedium GxcU  TTWYQDKRLGDIFTKMAPYLKVYTEYCGNYDKAVARLKQKSDESRDLQMFLKRVTSENSFGLDLNSLLVTPIQRIPRYKLLLQSLIQLTPVEFSDYPTLT  200

D discoideum GxcU   KALENVALVADHINESIREKQNSEKILTIQKRFTGQCPPLLAPLRTFIREGSLTKVCRKDHKKRWFILFSDALVYGNRIDTAVGNPIYKFHRLLPLSNTK  299
D intermedium GxcU  KALENVALVADHINESIREKQNSEKILTIQKRFTGQCPPLLAPLRTFIREGSLTKVCRKDHKKRWFILFSDALVYGNRIDTAVGNPIYKFHRLLPLSNTK  300

D discoideum GxcU   IANLDDTKHKNSFQLIHPIKSFTLIADTEQEKSSWMNSFSDAQNFLLRNEGSVARMNKQYRSINILKPKEEETVSTAPVWIPDSEAIQCMECTIKFTTIR  399
D intermedium GxcU  ISNLDDTEHKNSFQIIHPIKSFTLIADTEQEKLSWMSSFSDAQNFLSRNEGSVARMNKQY-TINILKQKEEEEVSTAPVWVPDSEAIQCMECTIKFTTIR  399

D discoideum GxcU   RRHHCRRCGNVVCGKCSEQKWTLDQYKKDARVCKTCYNYLSISSSRSNENNVSSSNN-----LSTPTSTFTATTATATAPFGTLT----DGASLADVSSD  490
D intermedium GxcU  RRHHCRRCGNVVCGKCSEQKWTLDQYKKDARVCKTCYNYLSISASRSNENNNNNNNNNSANSLSTPTTSTLTSTTTAATITSSITVTDISNASLADGSSD  499

D discoideum GxcU   SEGEDNSSSINDSNPDMPIINGLSSIQIDNTPVILNTVNPSPPLLSQPQPQQPQQPQLQPLQQPLQQPPQPPQPPQTNHKSFIATHTAYNKENSLKSTSP  590
D intermedium GxcU  SEGEDNSS-INDSNPDMPIINGLSSIQIDNTPVILNTVNSSPPLLSQQQQQQQQQQQQQQQQQ-QQQQQQQQQSVGHKSFIATHTAYNKENSLKSTSP  597

D discoideum GxcU   SSSFIETSTSTSASTSTSTSTSTSTSTSTSTSTSTSNSTSNSTLTPGPPMLSKSNAGNSSLLDFCKSYIQNSPNVSPAKVEKHKHSSSSSSASNSPMQ  690
D intermedium GxcU  SASFIEIP-----STSTSTPTPATASTATTATTTTSTT----------TPPMLSKSNAGNSSLLDFCKAYIQNSPSVSPAKVEKHKHSL---SASNSPMQ  679

D discoideum GxcU   SRTNLDIPS---TTTTSTSKGSISPTQSSESIVLPELKPITHDPLVTSNNSSSTLKVNQPIQHTNSSSKLNVIVSTPQTTQTTENNVNNTNTNTTTTNNN  787
D intermedium GxcU  SRSNLDIPSSTTTTTTTTTTGSISPNRSSESIVLPELKPITNDPLVTSNSSSSTLKVNQPIQHTNSSSKLNV---LPQTTQTLENS--------------  762

D discoideum GxcU   NNNNNNNNNNNNNNNNNNNNNNNNNSDVSSPKPTTKSLPPTPPTKKLPPTPPPKPLPPQPTLPQQPTLPPQPNLPPQPNLPPQPNLPPQPNLPQ  887
D intermedium GxcU  ------------SNISNGNNNSNNNNDVSSPKPTTKSLPPTPPTKKLPPTPPPKPLPPQPTLPQHPQ-------------------------QLQQ  821

D discoideum GxcU   QPDLSQPPPQQQQQPTLDQAPPRPPKPNQDSRSQSFLCISSTVNSTPPTPPPKRIISTHPANTSFIYQPQQYVSSPNINNVPPPLPPPRIKSQAPIQRD  986
D intermedium GxcU  PQQII---PQQQQQ--SDQAPPRPPKPNQD-RSQSSNCISSTVNSTPPTPPPKRIVSSYPANTSFIYQPQQHVTSPTLNNVPPPLPPPRIKTQSPIQRD  914
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RhoGEF   PH   FYVE

A Growth rate

B

C CV fusion rate
1h 1:1 Osmotic stimulus

D Labelling of CVs *prior* to CV collapse