RNAi screening of *Drosophila (Sophophora) melanogaster* S2 cells for ricin sensitivity and resistance

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Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; PDI, protein disulfide isomerise; RTA, ricin A chain

Abstract

The ribosome-inhibiting toxin ricin binds exposed β1→4 linked galactosyls on multiple glycolipids and glycoproteins on the cell surface of most eukaryotic cells. After endocytosis, internal cell trafficking is promiscuous, with only a small proportion of ricin proceeding down a productive (cytotoxic) trafficking route to the endoplasmic reticulum (ER). Here, the catalytic ricin A chain traverses the membrane to inactivate the cytosolic ribosomes, which can be monitored by measuring reduction in protein biosynthetic capacity or cell viability. Whilst some markers have been discovered for the productive pathway, many molecular details are lacking. To identify a more comprehensive set of requirements for ricin intoxication we have developed an RNAi screen in Drosophila S2 cells, screening in parallel the effects of individual RNAi treatments alone and when combined with a ricin challenge. Initial screening of 806 gene knockdowns has revealed a number of candidates for both productive and non-productive ricin trafficking including proteins required for transport to the Golgi, plus potential toxin interactors within the ER and cytosol.

Keywords: Ricin, RNAi, S2 cells, screen, PDI, ERAD

INTRODUCTION

The plant toxin ricin binds exposed cell-surface β1→4-linked galactosyls on surface components of mammalian cells via its B chain (RTB), entering the cells when these components are endocytosed. A small proportion of endocytosed ricin traffics to the endoplasmic reticulum (ER)[1]. Here the toxic A chain (RTA) is released from RTB by protein disulphide isomerise (PDI)[2], exposing a C-terminal hydrophobic patch on RTA which interacts with the ER membrane[3]. Subsequently, RTA crosses (dislocates) the ER membrane, entering the cytosol where it gains a catalytic conformation, aided by molecular chaperones[4]. It then specifically depurinates a position in large ribosomal subunit 28S rRNA[5], resulting in loss of protein synthesis ability and ultimately, cell death.

Overall knowledge of the intoxication pathway remains sparse. For example, ricin may traffic through the Golgi stack[6], but if so, it is not via known routes[7]. A likely reason is ricin's promiscuous surface binding which promotes multiple pathways to the ER lumen. To gain further insight into ER events, we expressed RTA in the ER lumen of *Saccharomyces cerevisiae*[8], from where it dislocates. Dislocation requires engagement with COPII-interacting p24 proteins, leading to Golgi trafficking and subsequent ER return. RTA then utilises the integral membrane HRD ubiquitylation complex of the ERAD (ER associated protein degradation) machinery that clears the ER of misfolded proteins, targeting them to the cytosolic proteasomes for destruction. However, RTA dislocates independently of ubiquitylation. Dislocated RTA then avoids the proteasomal core and the final destructive steps of ERAD[8].

Whilst this provides clues to RTA dislocation in mammalian cells, there are significant differences between yeast and mammalian systems: for example, yeast lacks the ER folding sensor UDP-glucose glycoprotein glucosyltransferase. Furthermore, since yeast lacks β1,4 galactosyltransferases, it does not express ricin receptors, so cannot be probed for trafficking requirements that lie upstream of the ER dislocation step. We therefore examined a genetically tractable higher eukaryote and describe here the establishment of an RNAi screen of *Drosophila* S2 cells to probe for all the requirements of ricin intoxication.

MATERIALS AND METHODS

RNAi library construction

The Expression Arrest™ RNAi library releases 1.0 and 2.0 were purchased as dsDNA templates in 96 well format (Open Biosystems)[9]. T7 polymerase was generated and purified as previously described[10]. *In vitro* transcription reactions were performed in a 20 µl volume reaction with 3 µg DNA template, 5 mM rNTPs, 0.015 $U.\mu l^{-1}$ yeast inorganic pyrophosphatase (Sigma) and 0.2 $U.\mu l^{-1}$ RNasin in a transcription buffer (30 mM HEPES (pH7.8), 100mM Potassium Glutamate, 15 mM Magnesium acetate, 25 mM EDTA, 1 mM DTT). Activity of T7 polymerase was assessed using these conditions and an optimal concentration per reaction employed for the library synthesis. Reactions were incubated at 37°C for 4h. RNAi was then diluted 5x by the addition of DEPC treated H_2O. Yield was assessed by agarose gel electrophoresis.

Growth of S2 cells and cytotoxicity assays

S2 cells were maintained in Drosophila-SFM (Invitrogen) containing 18 mM glutamine (Sigma), in rotating flasks (150 rpm, 28ºC). For cytotoxicity experiments, cells were seeded into 96-well plates (15,000 cells/well) and grown (3d) and a range of concentrations of ricin were added. After 24h, cell viability was assayed using MTS reagent (Promega) and a Mithras LB940 multimode reader (Berthold Technologies, Bad Wildbad, Germany). For RNAi screening experiments, cells were seeded into pairs of wells, one containing specific dsRNAi (750 ng/well) and the other an equivalent volume of water. After 3d, ricin was applied and cell viability was measured.

Western Blot

Cells were seeded (375,000 cells/well) into 2 wells of a 6-well plate, one containing specific RNAi targeting torp4a and the other an equivalent volume of water, and grown for 3d. After gentle centrifugation (100 x g, 5 min), extracts were taken by resuspending the cell pellets in 0.5 ml cold 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1% Triton X-100 containing protease inhibitor cocktail (Roche). Cell debris was removed (10,000 x g, 1 min) and protein concentrations of the soluble extracts were determined by colorimetric assay (Bio-Rad). Samples (10 µg) were separated by SDS-PAGE and immunoblotted. Torp4a protein was revealed by serial probing with

Rabbit anti-torp4a antibodies and peroxidise-conjugated anti-Rabbit antibodies followed by ECL (GE Healthcare) development.

RESULTS AND DISCUSSION

Adult Drosophila and S2 cells are sensitive to ricin

Fruit flies might be sensitive to ricin challenge since they express β1,4-galactosyltransferases and their ribosomes are sensitive to RTA[11]. Isogenised wild type Canton-S flies were starved (8h) to force a feeding response and were then fed once with 1% sucrose (controls) or with 1% sucrose containing ricin, followed by a daily maintenance diet of 1% sucrose. *Drosophila* show dose-dependent sensitivity to ricin; most died 4d after an initial toxin feed of 4 $\mu g.ml^{-1}$ ricin (Fig. 1A). By day 5 all surviving flies fed with this dose showed uncoordinated movements; none recovered from CO_2 anaesthesia.

Drosophila S2 cells are also sensitive to a 24h challenge with ricin (Fig. 1B). Addition of galactose during ricin challenge gave a dose-dependent protective effect (Fig. 1C). Thus, intoxication of fly cells depends upon galactose binding as it does in mammalian cells.

Establishing screening conditions

When S2 cells seeded at 15,000 cells per well in 96-well plates were grown for 4d, cells in the outer wells of a plate grew more slowly than those in the central wells (Fig. 2A). Cells were therefore grown in the central wells only, filling the outer wells with sterile water, resulting in optimal uniform growth.

A number of genes were selected to test RNAi conditions, along with 'scrambled' RNAi generated from a random arrangement of nucleotides from the sequence encoding human syntaxin 16. Cells grown (3d) in the presence of dsRNA were treated (24h) with a range of ricin concentrations. Golgi Tango7 RNAi did not alter the response to ricin challenge (Fig. 2B): similarly dsRNAs directed against the ER ribophorin, the cytosolic TER94 and 'scrambled' were ineffective (not shown). When assays were performed without ricin challenge, some RNAi treatments had clear growth effects (Fig. 2C), showing that for each RNAi knockdown screened with ricin there should be a coeval control lacking ricin.

To determine the screening concentration of ricin we treated cells with increasing doses of ricin (Fig. 3) and modelled the effects of RNAi that would provide 2-, 5- and 10-fold protection (P) or sensitisation (S). At a dose of 1 $\mu g.ml^{-1}$ ricin, protective RNAi effects of 2-fold or less would not be measurable, whereas after treatment with 62.5 $ng.ml^{-1}$ ricin, sensitising RNAi effects would be most easily measured. We chose 0.25 $\mu g.ml^{-1}$ ricin for screening, biasing expected results towards protective effects of 2-fold or more whilst still being able to recognise sensitising effects.

Preliminary screening of a library of individual RNAi molecules

Cells were grown in pairs of wells, both containing the same specific RNAi. One of the pair was then treated with ricin, and subsequent viability was measured. Screening plates also contained 6 control wells without RNAi treatment and a further 6 without RNAi treatment subsequently challenged with ricin. Values from control wells on each plate were used to determine a Z'-factor[12]. Plates that generated a Z'-factor of greater than 0.5 were considered for further evaluation. Of 34 plates screened, only one failed this test.

Initial screening of 96 randomly selected RNAi treatments is shown in Fig. 4A. Most RNAi treatments gave low MTS signals. Results are displayed as a scatter, plotting the relative effect of the combined RNAi/ricin treatment *versus* the relative effect of RNAi alone (Fig. 4B). Completely protective RNAi treatments would be expected along the line of unity connecting the (-) 'cells alone' control to the origin; ineffective RNAi treatments should lie along the line connecting the (+) 'cells plus ricin' control to the origin; protective RNAi treatments should accumulate in the segment between these lines and sensitising RNAi treatments should collect in the segment between the (+)-origin line and the abscissa. A few RNAi treatments sat outside these ranges with ricin-treated samples growing more strongly than non-treated cells. These false positives were from highly toxic RNAi treatments, reflecting difficulties in accurate measurement of small MTS signals.

For each RNAi treatment pair, a standard z-score was determined - the difference between treatment value (relative growth (%) after ricin treatment compared to treatment with RNAi alone) and the mean value of all treatments divided by the SD of all the treatment values. These are presented in a scatter *versus* relative

growth after individual RNAi treatment (Fig. 4C). Practical considerations limit accuracy of cytotoxicity curves where the RNAi-treated but non-ricin-treated controls give an MTS signal of about a quarter of that of non-RNAi treated controls, allowing us to reject 541 treatments (grey circles). Since RNAi treatment reduces rather than abolishes expression of target genes, sensitivity changes to ricin might be small. Furthermore, promiscuous binding/trafficking and multiple cytosolic interactions of ricin lead to small changes after interfering with genes controlling toxicity[1; 4]. Rather than use a z score threshold of 2 or 3, we therefore adopted an unusually low threshold of 1, at the risk of increasing the false positive rate, to ensure capture of such small changes. This led us to reject only those 182 treatments that lay between 0 and 1 SD from the mean score (white circles), leaving 45 RNAi treatments from an initial screening population of 806 that reflect potentially sensitising and protective RNAi treatments (black circles).

Confirmation of selected targets

Table 1 shows candidate 'hits' with human orthologs, ranked according to z-score. A selection is marked in Figs. 4A, B, C and D. Supplementary Table S1 shows candidates with no known human orthologs: most appear to have only arthropod orthologs, and may be uninformative.

CG5809 encodes a PDI family member. In mammalian cells PDI reduces the interchain disulphide bond between RTA and RTB[2]. We tested a different RNAi against PDI (from the Sheffield RNAi Screening Facility, UK), confirming that knockdown leads to protection against ricin in flies (Fig. 4D, upper panels).

CG6699 encodes the essential β'-COP subunit of the COPI coatomer complex which binds p24 cytosolic tails, allowing Golgi-to-ER transport of p24 proteins. Knockdown by alternative RNAi confirmed reproducibility (Fig. 4D). CG31787 encodes a family member of the fly p24 proteins, Type I transmembrane proteins with ill-defined roles in Golgi-ER cargo transport[13]. Its yeast ortholog is Erp2p, which promotes transport of RTA to the Golgi prior to recycling and dislocation[8]. Thus in fly cells, entry to the Golgi from the ER may require a specific interaction with a p24 protein. The

protective effects of RNAi against its expression and against expression of β'-COP in fly cells point to ER-Golgi cycling of RTA as a common feature of ricin intoxication.

The cytosolic fate of RTA is controlled by ubiquitin signals[4]. To test reproducibility for sensitising RNAi 'hits', two different RNAi molecules against the low z-score ubiquitin ligase-encoding archipelago ('arch', Fig. 4B), were compared (Fig. 4D, lower panels). Both sensitised cells slightly to ricin. Examining the remaining sensitising 'hits' revealed CG3024 (torp4a, an AAA-ATPase with a role in protein folding in the ER lumen, Fig. 4B) as a presumptive ER modulator of ricin cytotoxicity. Substantial torp4a knockdown gave only a modest (1.4 fold) sensitising effect (Fig. 4E), a minor effect consistent with low confidence in designating this a 'hit' from its z-score position in Fig. 4C.

We have designed and performed a preliminary screen of approximately 6% of *D. melanogaster* genes by RNAi knockdown, controlling in parallel for the effects of RNAi alone. If our screen had tested only specific RNAi treatment with subsequent ricin challenge, then from the first 96 RNAi treatments in Fig. 4A, some of the false positives and only RNAi against the p24-encoding CG31787 would have been identified as potentially protective, since these are the sole examples that gave a signal greater than that of ricin-treated control cells. The growth inhibitory effects of RNAi treatments against PDI and β'-COP would have led to the erroneous interpretation that reduced levels of these lead to ricin sensitivity. Indeed, the majority of RNAi treatments would be deemed to be highly sensitising, giving signals substantially lower than ricin-treated controls. A similar strategy that included testing the effects of gene depletion alone[14] underscores the need for inclusion of such a counter screen, which broadens the dynamic range and allows us to identify false positives more easily, thus improving the ability to identify likely candidates for genes involved in the ricin intoxication process.

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Table 1. Candidate RNAi treatments with human orthologs that influence ricin toxicity. Bold type indicates genes tested with two different RNAi molecules.

Target gene	name	Human ortholog	Candidate role
z>3			
CG8428	spinster	spinster homolog 1	endocytosis
3>z>2			
CG11184	Upf3	UPF3 regulator of nonsense transcripts homolog B (yeast)	gene silencing by miRNA
CG30429	none	MORN repeat containing 3	not known
CG10302	bicoid stability factor	leucine-rich PPR-motif containing	mRNA 3'-UTR binding
CG30338	none	RWD domain containing 2B	not known
CG31683	none	phospholipase A2, group XV	phospholipase
CG10078	Phosphoribosyl amidotransferase 2	phosphoribosyl pyrophosphate amidotransferase	nucleoside metabolic process
CG5809	**CaBP1**	**protein disulfide isomerase family A, member 6**	**reduction of ricin intra-chain disulphide**
CG31787	none	ERP2 (yeast)	ER to Golgi transport
CG3570	none	UPF0532 protein C7orf60	not known
CG6699	**β'-coatomer protein**	**coatomer protein complex, subunit beta 2 (beta prime)**	**ER-to-Golgi transport**
CG8726	none	PX domain containing serine/threonine kinase	protein kinase
CG13708	none	leucine rich repeat containing 49	not known
-2<z<-1			
CG3024	Torp4a	torsin family 1, member A (torsin A)	chaperone mediated protein folding

CG18654	none	diacylglycerol kinase, beta 90kDa	diacylglycerol kinase
CG5189	none	roadblock domain containing 3; Rab25	Golgi apparatus; endosome?
CG2173	Rs1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	RNA helicase; ribosome biogenesis
CG3766	scattered	vacuolar protein sorting 54 homolog	Endosome/Golgi transport and sorting
CG33008	none	transmembrane protease, serine 4	serine-type endopeptidase
CG14396	Ret oncogene	ret proto-oncogene	signal transduction
CG5403	retained	AT rich interactive domain 3A (BRIGHT-like)	transcription activator
CG15010	**archipelago**	**F-box and WD repeat domain containing 7**	**ubiquitin-protein ligase**
-3<z<-2			
CG4046	Ribosomal protein S16	ribosomal protein S16	structural constituent of ribosome
CG11079	none	5,10-methenyltetrahydro-folate synthetase (5-formyltetrahydrofolate cyclo-ligase)	unknown
CG3029	orange	adaptor-related protein complex 3, sigma 2 subunit	intracellular protein transport

Figure legends

Figure 1. Flies and S2 cells are sensitive to ricin. A). Starved adult flies were fed with one meal of ricin at a range of concentrations in 1% sucrose, then fed daily with a maintenance diet of 1% sucrose, and scored for survival. **B).** Dose response of S2 cells to a 24h exposure to a range of concentrations of ricin. **C).** Dose responses of S2 cells to a 24h exposure to a range of concentrations of ricin in the presence of increasing concentrations of galactose.

Figure 2. Establishing screening conditions. A). Mean viabilities of cells grown on the outer wells (outer, n = 36), remaining wells (inner, n = 60) or in the inner wells (n = 60) of a plate whose outer wells were occupied with water (inner plus moat). Bars, +/- 1 S.D. **B).** S2 cells were grown for 3d in the presence or absence of RNAi against Tango7 and then treated for 24h with a range of concentrations of ricin prior to MTS assay. **C).** Relative MTS signals of S2 cells treated with RNAi only.

Figure 3. Modelling the screen to determine ricin dose. Upper: S2 cells were treated with a range of ricin concentrations and viability was measured by MTS assay, generating a cytotoxicity curve (open circles). This curve was then shifted to the right or the left to model likely results from 2-, 5- or 10-fold shifts in sensitivity (2XS, 5XS and 10XS sensitising shifts: 2XP, 5XP and 10XP protective shifts). Lower: vertical slices (dotted lines, upper panel) were used to model expected results of treating with a range of concentrations of ricin. Arrows, magnitude of maximal protective (P) or sensitising (S) effects.

Figure 4. Screening results. A). Left panel; MTS signals from initial screening of 96 RNAi treatments are displayed as pairs of bars (white, RNAi treatment alone; black, RNAi treatment with subsequent ricin challenge). A number of candidate hits are highlighted (arrows). Right panel: corresponding ricin-treated controls (no RNAi treatment). **B).** Signals from **A** plotted in scatter format, defining the sectors into which protective and sensitising hits are likely to fall. (-), cells alone; (+) cells treated with ricin. **C).** Standard z-scores plotted versus relative growth of RNAi treated cells for all 768 RNAi treatments tested. Grey circles: treatments rejected because the RNAi signal alone was 25% or less that of non-RNAi-treated cells. White circles: treatments rejected that lie within 1 SD from the mean value. Black circles: 45 remaining candidate treatments that may influence ricin cytotoxicity. **D).** Testing alternative RNAi treatments: upper panels, RNAi treatments against PDI and β'-COP taken from **B** (RNAi 1, left) and from different RNAi treatments targeted against the same genes (RNAi 2, right); lower panels, RNAi 1 (left panel) from **B** against archipelago (arch) and an alternative RNAi 2 against the same gene (right panel). **E).** Upper panel: cell extracts (10μg) of S2 cells treated or not (ctl) with torp4a RNAi were electrophoresed, immunoblotted and probed for torp4a protein. *, cross-reacting protein. Approximate migration of size markers are shown on the left. Lower graph: S2 cells treated or not with torp4a RNAi were subsequently treated with ricin and viabilities were determined by MTS assay.

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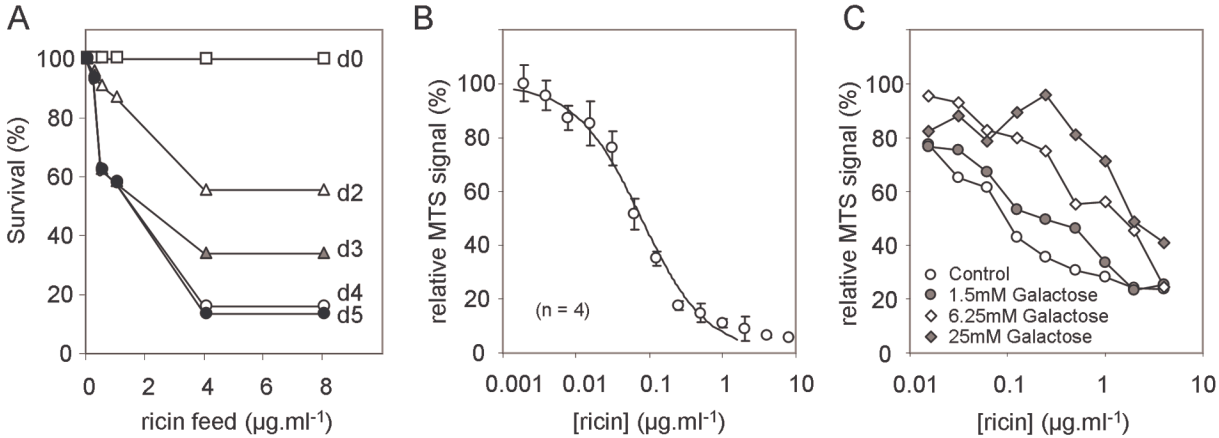
Supplementary Table S1: Candidate RNAi treatments with no obvious human orthologs

Target gene	name	Potential role
2>z>1		
CG18659	none	not known
CG17064	mars	regulation of mitotic cell cycle
CG9858	cricklet	carboxylesterase
CG30340	none	G-protein coupled receptor
CG33503	Cyp12d1-d	cytochrome P450
CG32249	none	signal transducer
CG32335	none	hydrolase activity, acting on ester bonds
CG8652	UDP-glycosyl-transferase 37c1	glucuronosyltransferase
-2<z<-1		
CG11163	none	cation transport
CG14746	PGRP-SC1a	innate immune response
CG5781	none	not known
CG30491	none	short-chain dehydrogenases/reductases family (SDR)
CG30090	none	serine-type endopeptidase
CG10972	pickpocket 12	sodium channel
CG33461	none	serine-type endopeptidase
CG30082	none	not known
CG30268	none	not known
CG4302	none	glucuronosyltransferase
-3<z<-2		
CG32245	none	not known
CG3215	none	glycerol-3-phosphate dehydrogenase


A
Survival (%)
100
80
60
40
20
0
d0
d2
d3
d4
d5
0
2
4
6
8
ricin feed (µg.ml-1)
B
relative MTS signal (%)
100
80
60
40
20
0
(n = 4)
0.001
0.01
0.1
1
10
[ricin] (µg.ml-1)
C
relative MTS signal (%)
100
80
60
40
20
0
Control
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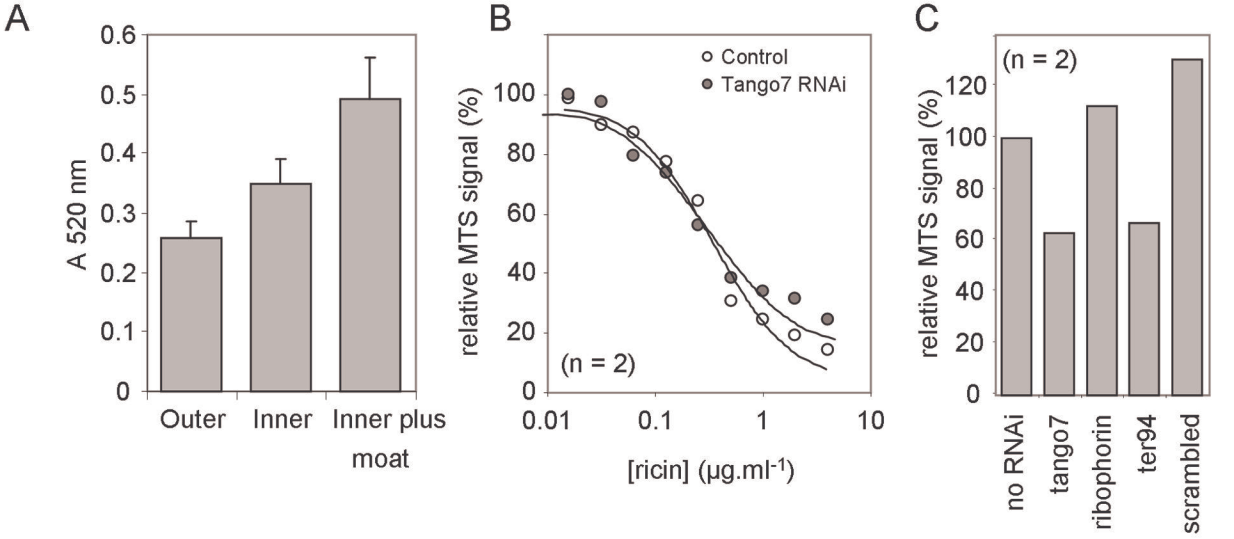
6.25mM Galactose
25mM Galactose
0.01
0.1
1
10
[ricin] (µg.ml-1)


A
A 520 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
Outer
Inner
Inner plus moat
B
relative MTS signal (%)
100
80
60
40
20
0
Control
Tango7 RNAi
(n = 2)
0.01
0.1
1
10
[ricin] (µg.ml-1)
C
relative MTS signal (%)
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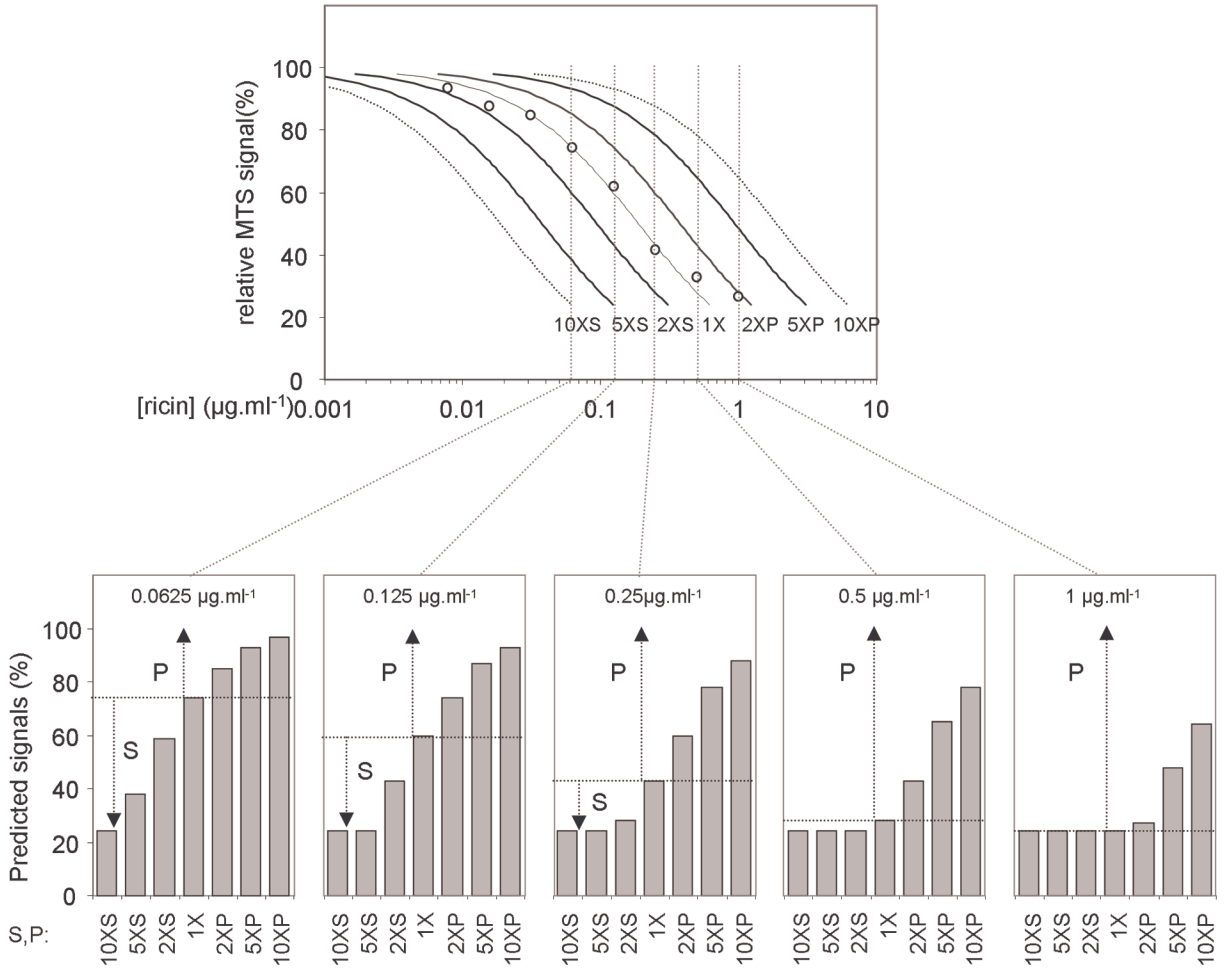
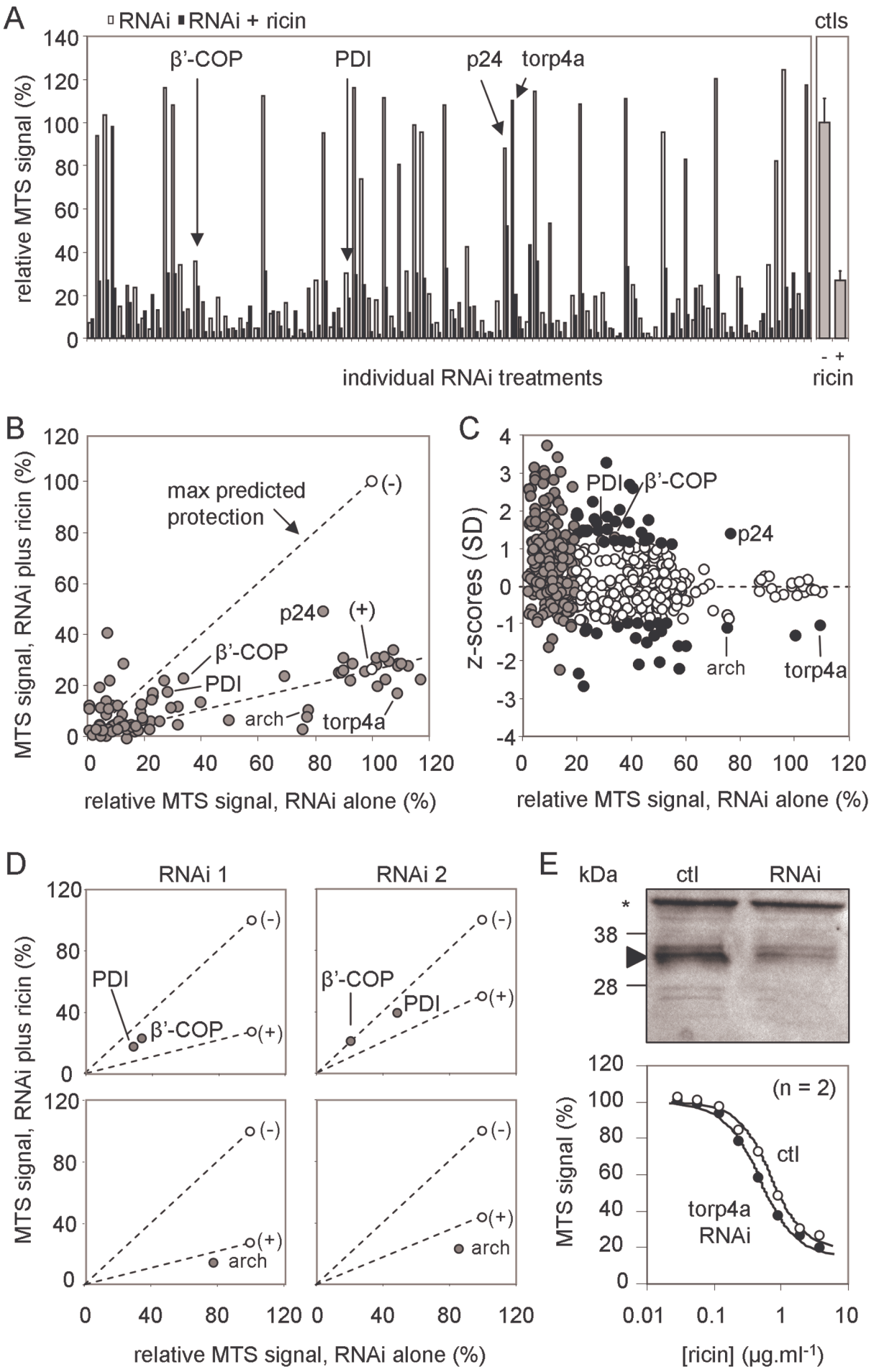
100
80
60
40
20
0
(n = 2)
no RNAi
tango7
ribophorin
ter94
scrambled


relative MTS signal(%)
100
80
60
40
20
0
10XS 5XS 2XS 1X 2XP 5XP 10XP
[ricin] (µg.ml-1)
0.001
0.01
0.1
1
10
0.0625 µg.ml-1
0.125 µg.ml-1
0.25µg.ml-1
0.5 µg.ml-1
1 µg.ml-1
Predicted signals (%)
P
S
S,P:
10XS 5XS 2XS 1X 2XP 5XP 10XP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RNAi
RNAi + ricin
ctls
β'-COP
PDI
p24
torp4a
relative MTS signal (%)
individual RNAi treatments
- +
ricin
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max predicted protection
(-)
(+)
p24
β'-COP
PDI
arch
torp4a
MTS signal, RNAi plus ricin (%)
relative MTS signal, RNAi alone (%)
C
PDI
β'-COP
p24
arch
torp4a
z-scores (SD)
relative MTS signal, RNAi alone (%)
D
RNAi 1
RNAi 2
PDI
β'-COP
arch
MTS signal, RNAi plus ricin (%)
relative MTS signal, RNAi alone (%)
E
kDa
ctl
RNAi
38
28
(n = 2)
ctl
torp4a RNAi
MTS signal (%)
[ricin] (μg.ml-1)