SUPPLEMENTARY INFORMATION

Differential regulation of elastic fiber formation by fibulins -4 and -5

Rawshan Choudhury[1#], Amanda McGovern[1#], Caroline Ridley[2#], Stuart A. Cain[1], Andrew Baldwin[1], Ming-Chuan Wang[1], Chun Guo[1], Aleksandr Mironov Jnr.[1], Zoe Drymoussi[1], Dorothy Trump[2], Adrian Shuttleworth[1], Clair Baldock[1] and Cay M. Kielty[1]*
[#]*Joint first authors*

[1]Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, and [2]Faculty of Medical and Human Sciences, University of Manchester, UK.

LEGENDS TO SUPPLEMENTARY FIGURES

FIGURE S1
SDS-PAGE analysis of recombinant fibulins -4 and -5, fibrillin-1 and LOX

The following proteins were expressed and analyzed by SDS-PAGE in the presence or absence of 10 mM dithiothreitol (R or NR, respectively), by Coomassie blue staining: full-length fibulin-4 (F4), C-terminally truncated fibulin-4 (tF4), the N-terminal half of fibulin-4 (nF4) and the five central cbEGFs (eF4); full-length fibulin-5 (F5) without or with two cutis laxa mutations ($F5_{C217R}$ and $F5_{S227P}$), and domain pair fragments; fibrillin-1 fragments have previously been reported (not shown) (1-8) apart from a two-thirds fragment (tFib-1) which is shown by western blotting as a 250 kDa fragment using several anti-fibrillin-1 antibodies including (shown) fibrillin-1-specific RGD antibody (9); full-length lysyl oxidase (LOX). Treatment of F4 and tF4 with N-glycosidase F (PNGase F; indicated by arrows) resulted in slightly faster electrophoretic migration, indicating that both are N-glycosylated. Treatment of F5 and mutants, F5-E4+5, F5-E5+6 and F5-E6FC with N-glycosidase F also confirmed that they were N-glycosylated (not shown) (1,3). Fibrillin-1 fragments have been confirmed to be N-glycosylated.

FIGURE S2
Mass spectrometry of truncated fibulin-4 and fibrillin-1

(A) The peptide sequence coverage (shown in red text), as determined by mass spectrometry, of F4 and tF4. Truncated fibulin-4 tF4 lacks the FC domain and 6 preceding amino acid residues (for domain organization, *see* Fig. 1A).
(B) The peptide sequence coverage (shown in red text), as determined by mass spectrometry, of the two-thirds truncated fibrillin-1 fragment (tFib-1) (for domain organization, *see* Fig. 1B).

FIGURE S3
Multi-angle laser light scattering of human fibulins -4 and -5

Monomers of (i) fibulin-4 (F4) and (ii,iii) fibulin-5 (F5 and $F5_{S227P}$) that had been isolated by Superdex 200 gel filtration in HBS, were analyzed by MALLS in HBS. Samples eluting from the column passed through a Wyatt EOS 18-angle light scattering detector fitted with a 688 nm laser and an Optilab r-EX refractometer (*see* Table 1). F4 was monomeric in the absence of calcium, but formed dimers in the presence of calcium. Similarly, F5 and F5 mutant $F5_{S227P}$ were

monomeric in the absence of calcium, but dimeric in the presence of calcium (not shown; Jones *et al*, in preparation).

FIGURE S4

Molecular interactions of fibulin-5, fibrillin-1 and tropoelastin

(A) Solid-phase assays of biotinylated fibulin-5 fragments binding to immobilized tropoelastin. Three of the domain pair fragments, F5-E1+2, F5-E4+5 and F5-E6FC bound well to immobilized tropoelastin (K_Ds 332 nM, 452 nM and 965 nM, respectively). One representative experiment is shown. Data are shown with the negative (biotinylated F5 fragments only) control subtracted. Results are shown as the mean ± S.E. of triplicate values.

(B) Solid-phase curves showing soluble biotinylated fibulin-5 domain-pair fragments (F5-E1+2 or F5-E6FC) binding to immobilized PF1. These fibulin-5 fragments only bound N-terminal fibrillin-1. One representative experiment is shown. Data are shown with the negative (biotinylated F5 fragments only) control subtracted. Results are shown as the mean ± S.E. of triplicate values.

(C) BIAcore analysis of soluble tFib-1 (200 nM) (C-terminally truncated two-thirds fragment of fibrillin-1; see Figure S1)) binding to immobilized tropoelastin. Response difference is the difference between experimental and control flow cells, in response units (RU). Pre-incubation of F5 with tFib-1 neither inhibited nor stimulated the binding of fibrillin-1 to tropoelastin (not shown).

(D) Fibrillin-1 (tFib-1) inhibits soluble F4 interacting with immobilized tropoelastin. BIAcore analysis of the interaction of soluble F4 with immobilized tropoelastin, showing that pre-incubation of F4 with tFib-1 (C-terminally truncated two-thirds fragment of fibrillin-1; see Figure S1) inhibits F4 binding to tropoelastin.

FIGURE S5

Elastic fiber molecules expressed by dermal fibroblasts, ARPE-19 cells and RFL6 cells

(A) (i) RT-PCR analysis of the expression of fibulins -4 and -5 by ARPE-19 cells. Fibulin-4 was expressed at moderate levels, but fibulin-5 expression was very low. (ii) Fibulin-4 was knocked down in ARPE-19 cells using siRNA. Quantitative PCR (qPCR) and immunoblot analysis (not shown) showed 70% knockdown of fibulin-4.

(B) The expression of N-terminally V5-tagged human elastin (E) by ARPE-19 cells, after retroviral induction, was detected on blots by anti-V5 antibody (Abcam) and by immmunofluorescence microscopy (red) using a polyclonal anti-elastin antibody (PR398, Elastin Products Inc.), with DAPI (blue) staining of cell nuclei.

(C) Fibulin-5 was stably knocked down in RFL6 cells by retroviral shRNA. (i) RT-PCR and (ii, iii) qPCR, respectively, showed that fibulin-5 is depleted in the knockdown cells, compared to the scrambled control cells. qPCR also revealed that fibulin-4 is not altered in the fibulin-5 shRNA knockdown cells (not shown).

FIGURE S6

Effects of heparin on tropoelastin binding by fibulins -4 and -5

Heparin binds tropoelastin and influences its coacervation (Tu and Weiss, 2008). We examined whether heparin altered interactions of fibulins -4 and -5 with tropoelastin, by solid phase assays.

(A) (i) Pre-incubation of fibulins -4 or -5 with 10 nM or 100 nM heparin resulted in small but significant reductions in each fibulin binding to tropoelastin. A significant difference in binding of F4 or F5 to tropoelastin in the presence or absence of heparin was shown as ***, $p < 0.001$ (unpaired *t* test). (ii) Increasing concentrations of heparin inhibited fibulin-4 binding to tropoelastin, but concentration-dependent heparin effects on fibulin-5 binding were less clear. In each case, one representative experiment is shown. Data are shown with the negative (biotinylated F4 or F5 only) control subtracted. Results are shown as the mean ± S.E. of triplicate values.
(B) Pre-incubation of three fibulin-5 domain pair fragments that bind tropoelastin (see Fig. 2C) with heparin (both at 100 nM) resulted in significant reductions in fibulin binding to tropoelastin. Significant differences in binding of fibulin-5 domain pair fragments to tropoelastin in the presence or absence of heparin were shown as *, $p<0.01$; **, $p < 0.05$; ***, $p < 0.001$ (unpaired *t* test). In each case, one representative experiment is shown. Data are shown with the negative (biotinylated F5 fragments only) control subtracted. Results are shown as the mean ± S.E. of triplicate values.

References

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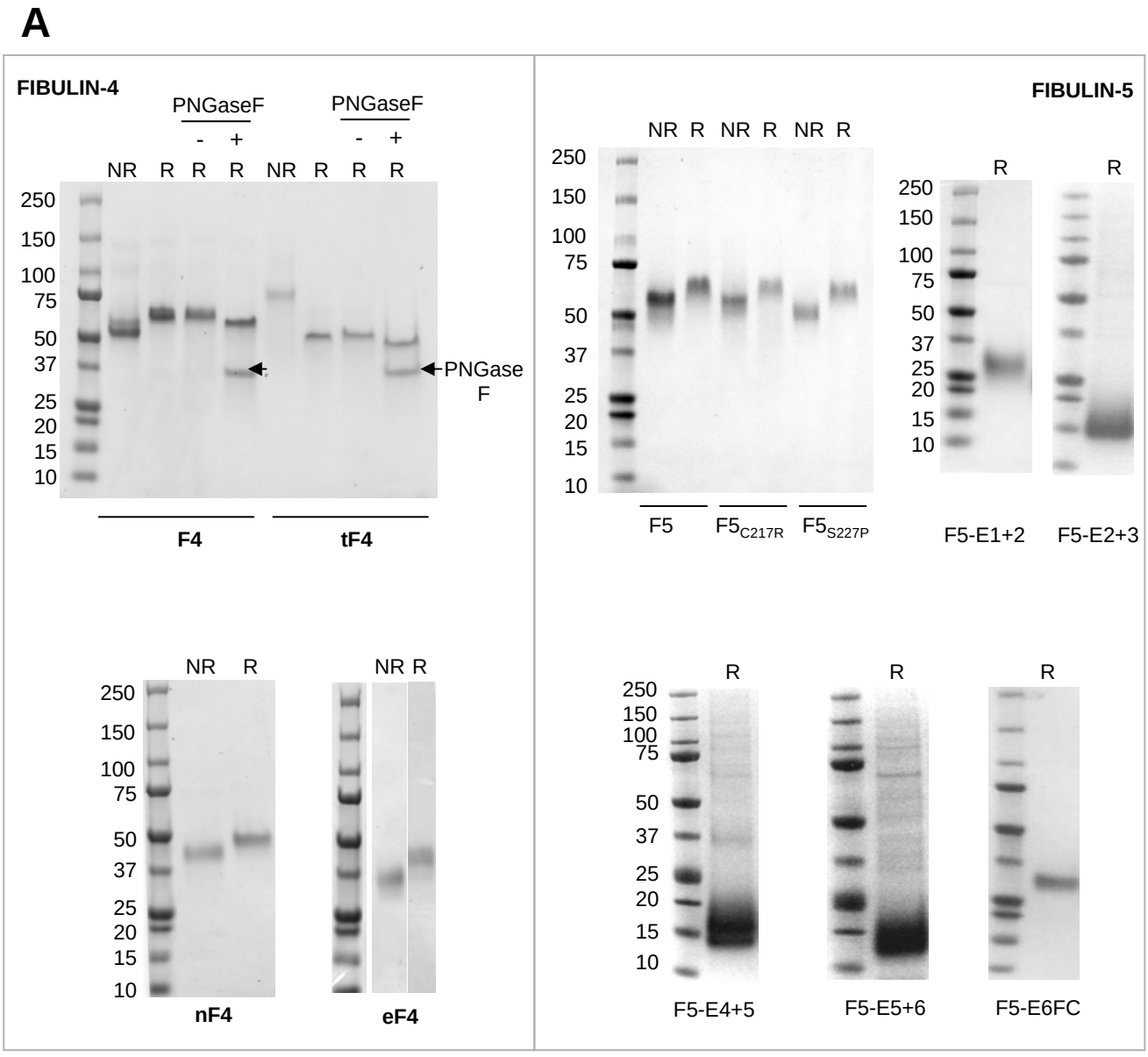
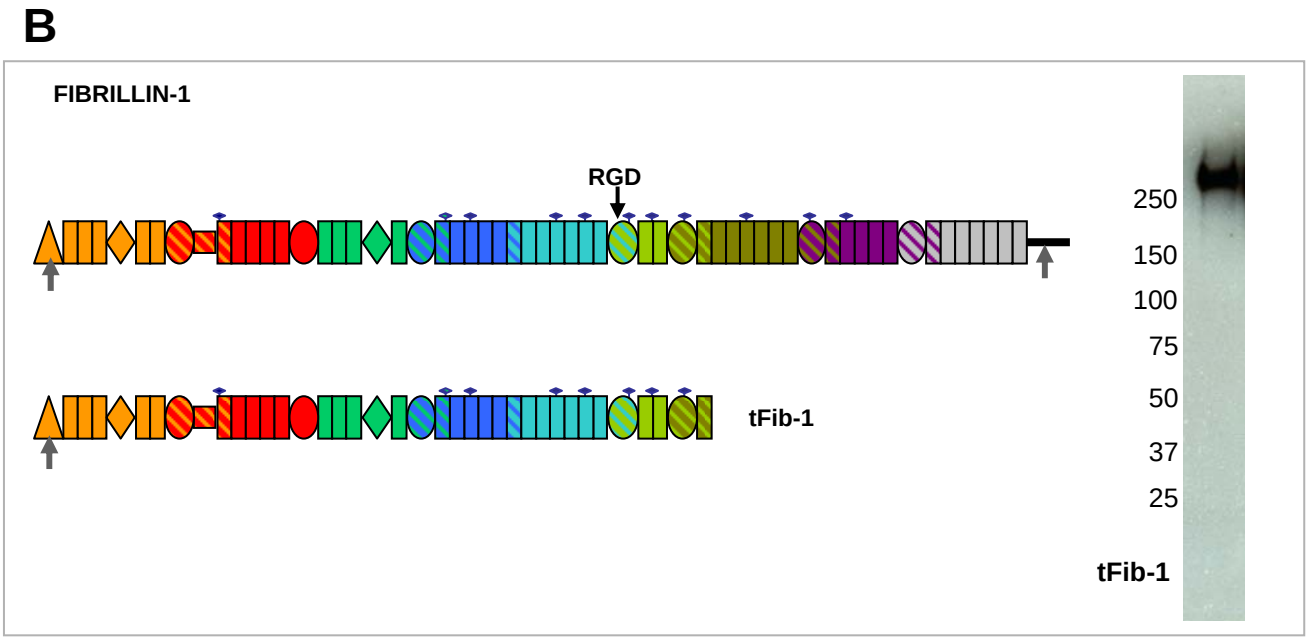
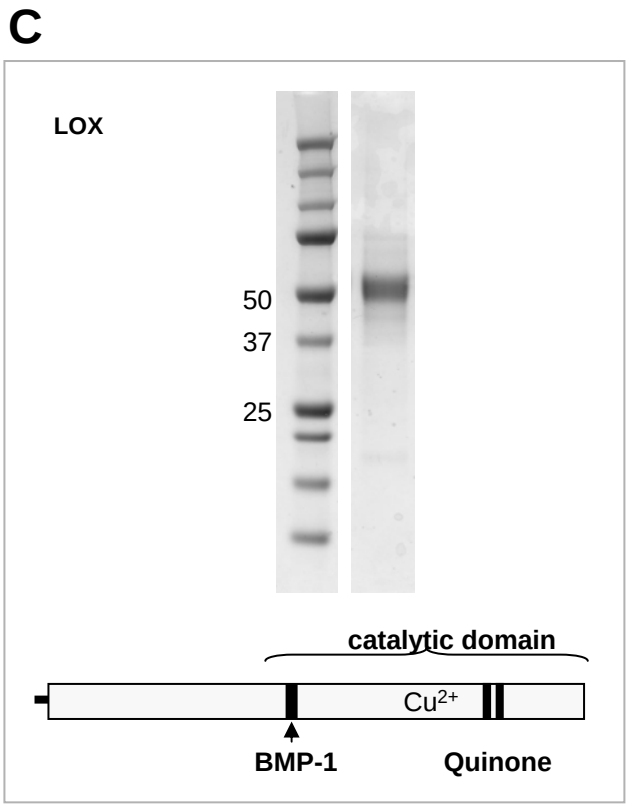
Supplementary Figure 1



Supplementary Figure 1





Supplementary Figure 2

A

Full-length Fibulin-4 (F4)

mlpcasclpg slllwallll llgsaspqds eepdsytect dgyewdpdsq
hcrDVNECLT IPEACKGEMK CINHYGGYLC LPRsaavind lhgegppppv
ppaqhpnpcp pgyepddqds cvdvdecaqa lhdcrpsqdc hnlpgsyqct
cpdgyrKIGP ECVDIDECRY RycqhrCVNL PGSFRcqcep gfqlgpnnrS
CVDVNECDMG APCEQRCFNS YGTFLCRCHQ GYELHRDGFS CSDIDECSYS
SYLCQYRCIN EPGRFSCHCP QGYQLLATRl cqdidecesg ahqcseaqtc
vnfhggyrCV DTNRCVEPYI QVSENRCLCP ASNPLCREQP SSIVHRYMTI
TSERSVPADV FQIQATSVYP GAYNAFQIRA GNSQGDFYIR QINNVSAMLV
LARPVTGPRE YVLDLEMVTM NSLMSYRass vlrltvfvga ytf

Truncated Fibulin-4 (tF4)

mlpcasclpg slllwallll llgsaspqds eepdsytect dgyewdpdsq
hcrDVNECLT IPEACKGEMK CINHYGGYLC LPRsaavind lhgegppppv
ppaqhpnpcp pgyepddqds cvdvdecaqa lhdcrpsqdc hnlpgsyqct
cpdgyrKIGP ECVDIDECRY RycqhrCVNL PGSFRcqcep gfqlgpnnrS
CVDVNECDMG APCEQRCFNS YGTFLCRCHQ GYELHRDGFS CSDIDECSYS
SYLCQYRcin epgrFSCHCP QGYQLLATRl cqdidecesg ahqcseaqtc
vnfhggyrCV DTNRCVEPYI QVSENRCLCP ASNPLCReqp ssivhrYMTI
TSERSVPADV FQIQATSVYP GAYNAFQIRA GNSQGDFYIR qinnvsamlv
larpvtgpre yvldlemvtm nslmsyrass vlrltvfvga ytf

Supplementary Figure 2

B

Truncated fibrillin-1 (tFib-1)

mrrgrlleia lgftvllasy tshgadanle agnvketras rakrr**GGGGH DALKGPNVCG SRYNAYCCPG WKTLPGGNQC**
IVPICRHSCG DGFCSRPNMC TCPSGQIAPS CGSRsiqhcn ir**CMNGGSCS DDHCLCQKGY IGTHCGQPVC ESGCLNGGR**c
vapnr**CACTY GFTGPQCER**d yr**TGPCFTVI SNQMCQGQLS GIVCTK**qlcc atvgr**AWGHP CEMCPAQPHP CRRGFIPNIR**
tgacqdvdec qaipglcqgg ncintvgsfe ckcpaghkln evsqkcedid ecstipgice ggectntvss yfck**CPPGFY**
TSPDGTRCID VRPGYCYTAL TNGRCSNQLP QSITKMQCCC DAGRCWSPGV TVAPEMCPIR atedfnklcs vpmvipgrpe
ypppplgpip pvlpvppgfp pgpqipvprp pveylypsre pprvlpvnvt dycqlvrylc qngrciptpg syrcecnkgf
qldlrgecid vdeceknpca ggecinnqgs ytcqcr**AGYQ STLTR**tecr**D IDECLQNGR**i cnngrcintd gsfhcvcnag
fhvtr**DGKNC EDMDECSIR**n mclngmcine dgsfk**CICKP GFQLASDGR**y ck**DINECETP GICMNGRCVN TDGSYRCECF**
PGLAVGLDGR VCVDTHMRst cyggykr**GQC IKPLFGAVTK** secccastey afgepcqpcp aqnsaeyqal cssgpgmtsa
gsdinecald pdicpngice nlrgtyk**CIC NSGYEVDSTG KNCVDINECV LNSLLCDNGQ CR**ntpgsfvc tcpk**GFIYKP**
DLKtcedide cesspcingv ck**NSPGSFIC ECSSESTLDP TK**ticietik **GTCWQTVIDG RCEININGAT LKSQCCSSLG**
AAWGSPCTLC QVDPICGKgy srikgtqced idecevfpgv ck**NGLCVNTR** gsfk**CQCPSG MTLDATGRIC LDIRLETCFL**
RYEDEECTLP IAGRhrmdac ccsvgaawgt eeceecpmr**N TPEYEELCPR** gpgfatk**EIT NGKPFFK**din eck**MIPSLCT**
HGKcrntigs fkcr**CDSGFA LDSEER**nctd idecrispdl cgr**GQCVNTP GDFECK**cdeg yesgfmmmk**N CMDIDECQRD**
PLLCRggvch ntegsyrcec ppghqlspni sacidinece lsahlcpngr cvnligk**YQC ACNPGYHSTP DR**lfcvdide
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ctdldecsng thmcsqhadc kntmgsyrcl ckegytgdgf tctdldecse nlnlcgngqc lnapggyr**CE CDMGFVPSAD**
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tviledidec qelpglcqgg k**CINTFGSFQ CRCPTGYYLN EDTR**vcddvn ecetpgicgp gtcyntvgny tcicppdymq
vnggnncmdm rrslcyrnyy adnqtcdgel lfnmtkkmcc csynigrawn kpceqcpips tdefatlcgs qrpgfvidiy
tglpvdidec reipgvceng vcinmvgsfr **CECPVGFFYN DK**llvcedid ecqngpvcqr naecintags yrcdckpgyr
ftstgqcndr necqeipnic shgqcidtvg sfyclchtgf ktnddqtmcl dinecerdac gngtcrntig sfncrcnhgf
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qfsggcqdin ecgsaqapcs ygcsnteggy lcgcppgyfr igqghcvsgm gmgrgnpepp vsgemddnsl speacyecki
ngypkrgrkr rstnetdasn iedqsetean vslaswdvek taifafnish vsnkvrilel lpalttltnh nryliesgne
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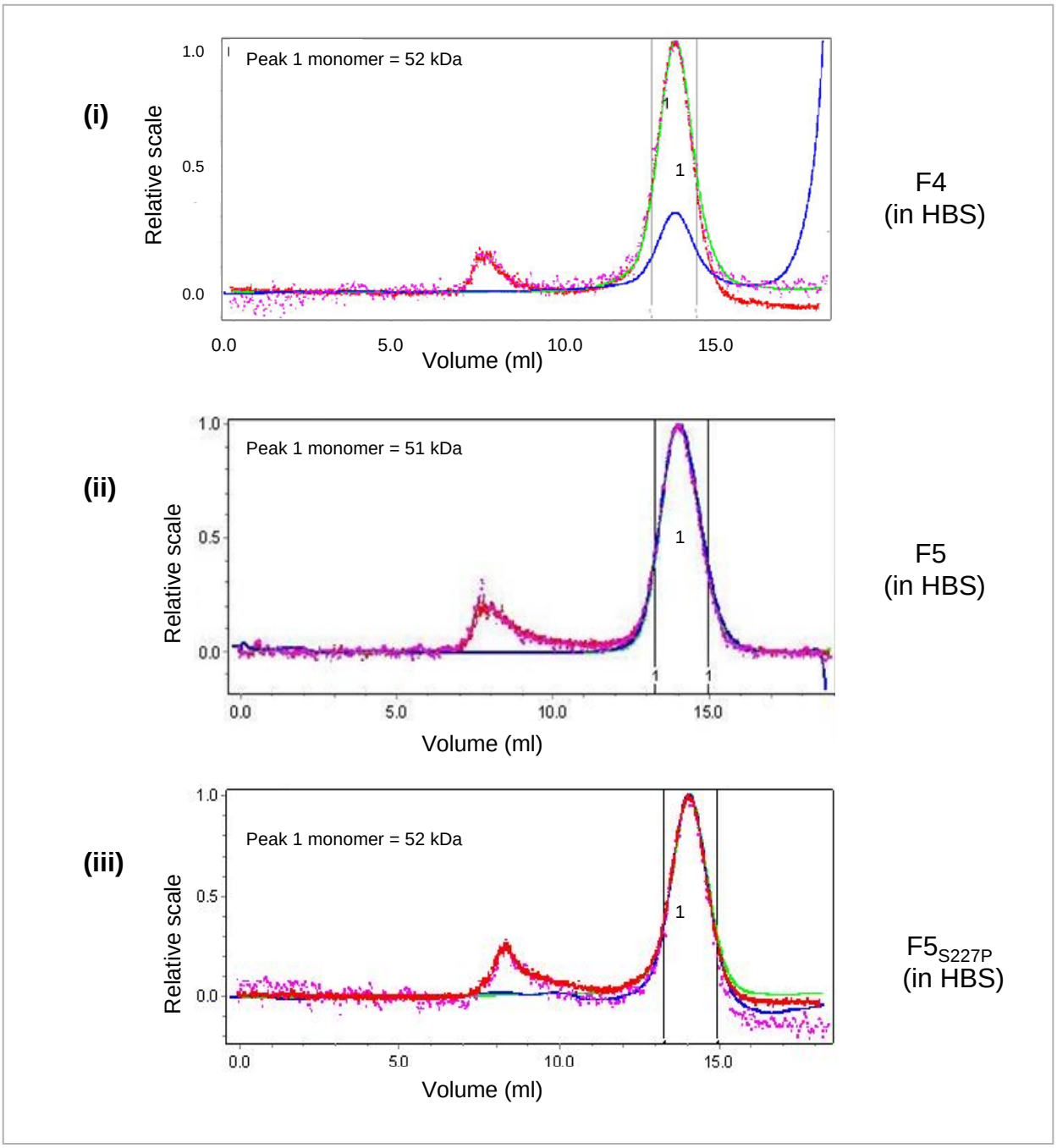
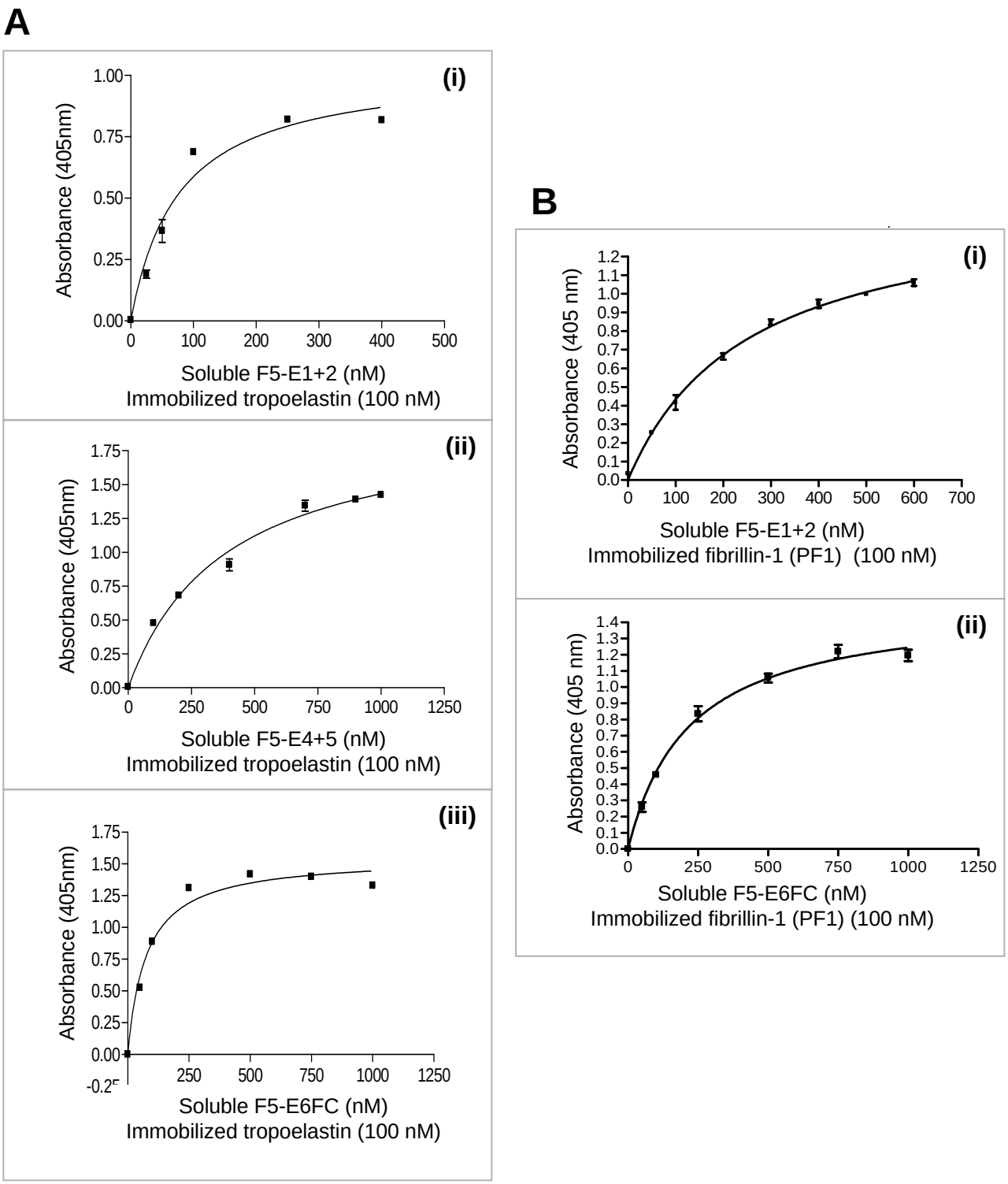
Supplementary Figure 3

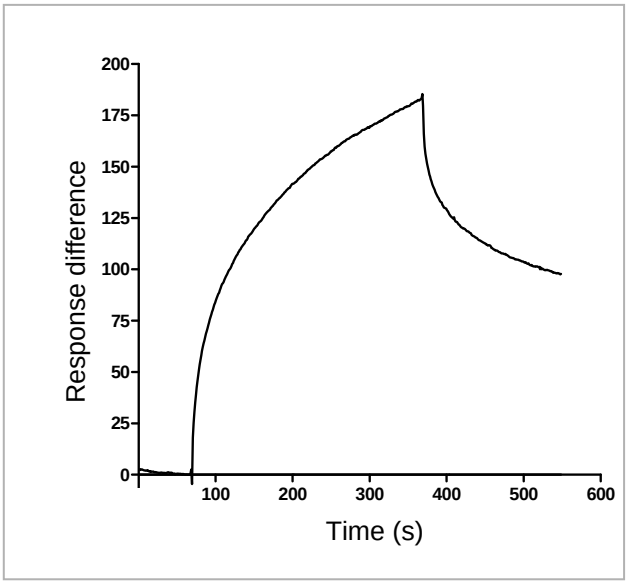


Supplementary Figure 4



Supplementary Figure 4

C



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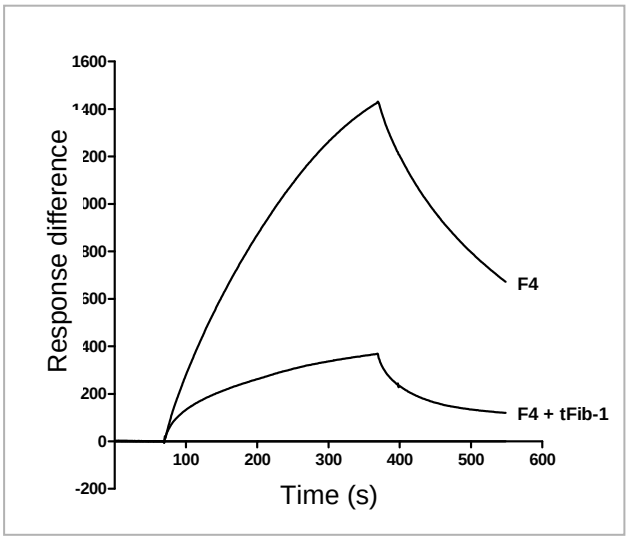

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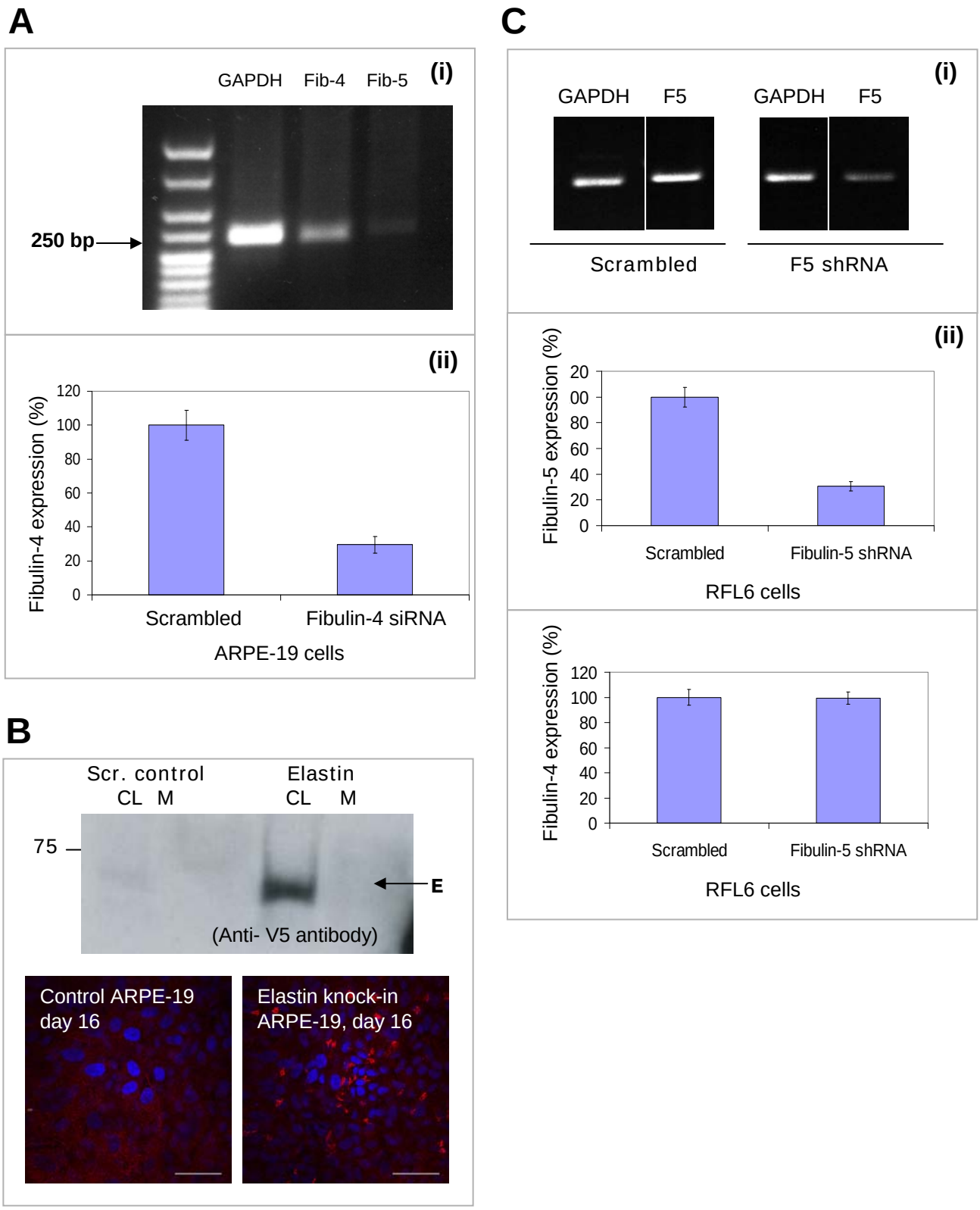

Supplementary Figure 6