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Supporting Information

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Supporting Information

for

Bipartite Tetracysteine Display Requires Site Flexibility for ReAsH Coordination

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Materials. The stringent and permissive ReAsH binding buffers were made fresh from 1 M Trizma hydrochloride solution (pH 8) obtained from Sigma-Aldrich, tris-(2-carboxyyethyl)-phosphine HCl (TCEP) from Pierce, NaCl was purchased from Sigma-Aldrich, 1,2 ethanedithiol (EDT) (purum grade) was from Fluka was obtained as a 0.5 M solution (pH 8.0) and dimethylsulfoxide (DMSO) was obtained from J.T. Baker. The ReAsH-EDT_2 (Figure S1B) used was synthesized as per the previously reported methods.[1-3]

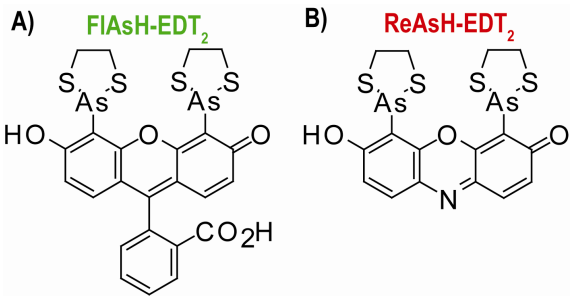

Figure S1. Chemical structures of A) FlAsH-EDT_2 and B) ReAsH-EDT_2.

Protein cloning, expression and purification of the p53 bipartite display variants. The pRSET-derived vector containing the p53 core domain (residues 94-312) with an N-terminal fusion of 6xHis/lipoamyl domain/thrombin cleavage sequence was obtained courtesy of Alan Fersht, Cambridge University. Cysteine mutations were introduced into the sequence using QuikChange Site-Directed Mutagenesis (Stratagene) with the following primers:

p53-1. P92C L93C P301C G302C

Primer Name	Primer Sequence (5' to 3')
P92C_L93C	5'-tgccgcgcggatcctgttgctcatcttctgtccc-3'
P92C_L93C antisense	5'-gggacagaagatgagcaacaggatccgcgcggca-3'
P301C_G302C	5'-cctcaccacgagctgccctgctgcagcatctaagcgagcactg -3'
P301C_G302C antisense	5'-cagtgctcgcttagtgctgcagcagggcagctcgtggtgagg-3'

p53-2. Y107C G108C D148C S149C

Primer Name	Primer Sequence (5' to 3')
Y107C_G108C	5'-ctaccagggcagctgctgtttccgtctgggc-3'
Y107C_G108C antisense	5'-gcccagacggaaacagcagctgccctggtag-3'
D148C_S149C	5'-gtgcagctgtgggtttgttgcacacccccgcccg-3'
D148C_S149C antisense	5'-cgggcgggggtgtgcaacaaacccacagctgcac-3'

p53-3. S116C G117C T123C

Primer Name	Primer Sequence (5' to 3')
S116C_G117C	5'-ggtttccgtctgggcttcttgcattgttgcacagccaagtctg-3'
S116C_G117C antisense	5'-cagacttggctgtgcaacaatgcaagaagcccagacggaaacc-3'
T123C	5'-gacagccaagtctgtgtgttgcacgtactcccct-3'
T123C antisense	5'-aggggagtacgtgcaacacacagacttggctgtc-3'

p53-4. S96C V97C M169C T170C

Primer Name	Primer Sequence (5' to 3')
S96C V97C	5'-tgccgcgcggatcctcatcttgttgcccttcccaga -3'
S96C V97C antisense	5'-tctgggaagggcaacaagatgaggatccgcgcggca -3'
M169C T170C	5'-atctacaagcagtcacagcactgctgcgaggttgtgaggcgctgcccc -3'
M169C T170C antisense	5'-ggggcagcgcctcacaacctcgcagcagtgctgtgactgcttgtagat -3'

p53-5. A129C L130C R290C K291C

Primer Name	Primer Sequence (5' to 3')
A129C L130C	5'-tgtgacttgcacgtactccccttgctgcaacaagatgttttgccaactg -3'
A129C L130C antisense	5'-cagttggcaaaacatcttgttgcagcaaggggagtacgtgcaagtcaca-3'
R290C K291C	5'-cacagaggaagagaatctctgctgcaaaggggagcctcaccacg -3'
R290C K291C antisense	5'-cgtggtgaggctccccctttgcagcagagattctcttcctctgtg-3'

Colonies from the QuikChange Mutagenesis were mini-prepped (Qiagen) and sequenced by using the T7 Promoter sequencing primer (W.M. Keck Facility at Yale). Plasmids with the correct sequence were transformed into Overexpress C41 (DE3) competent cells (Lucigen). Colonies were grown overnight in regular media (5 mL) supplemented carbenicillin (starter culture). The next day, starter culture (1 mL) was added to regular media (1 L) supplemented with carbenicillin. When the cultures reached an OD_{600} of 0.6, the cultures were induced at 25^{o}C with fresh Isopropyl β-D-1-thiogalactopyranoside (0.4 mM IPTG, Aldrich). The cultures were allowed to shake overnight at 25^{o}C, afterwards the cells were immediately pelleted. Lysis buffer (50 mL, buffer composition: 0.05 M Tris HCl, 0.15 M NaCl, 3.5 mM TCEP, 0.02 M Imidazole, and EDTA free complete protease inhibitor (Roche)) was used to resuspend the pellet. After cell lysis via sonication, the lysate was centrifuged for 20 mins at 15 000 rpm. The supernantant was incubated with washed Ni-NTA (2 mL) resin for 6 h and subsequently added to a PD-10 column. The resin was washed with 30 column volumes of wash buffer 1 (0.05 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP, 20 mM imidazole). And the protein was eluted with the elution buffer (0.05 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP, 250 mM Imidazole). The samples were then dialyzed into thombin cleavage buffer (0.025 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP). Digestion with biotinylated thrombin (Novagen) overnight at 4 ^{o}C was performed and followed by thrombin capture using the biotinylated thrombin capture kit (Novagen). After diluting the thrombin cleavage reaction two fold in FPLC buffer containing no NaCl (0.025 M Tris HCl, 3.5 mM TCEP), the p53 core domain was purified by cation exchange FPLC using a Hi Trap Q FF cation exchange column (Amersham Biosciences).

Protein cloning, expression and purification of the EmGFP bipartite display variants. The pRSET-EmGFP vector was purchased from Invitrogen. Cysteine muta-

tions were introduced into the sequence using QuikChange Site-Directed Mutagenesis (Stratagene) with the following primers:

EmGFP-1 (D19C, D21C, K26C, S28C)

Primer Name	Primer Sequence (5' to 3')
D19C_D21C	5'-ccatcctggtcgagctgtgcggctgcgtaaacggccacaagt-3'
D19C_D21C_antisense	5'-acttgtggccgtttacgcagccgcacagctcgaccaggatgg-3'
K26C_S28C	5'-cgacgtaaacggccactgcttctgcgtgtccggcgaggg-3'
K26C_S28C_antisense	5'-ccctcgccggacacgcagaagcagtggccgtttacgtcg-3'

EmGFP-2 (E34C, D36C, K41C, T43C)

Primer Name	Primer Sequence (5' to 3')
E34C_D36C	5'-cgtgtccggcgagggctgcggctgtgccacctacggcaag-3'
E34C_D36C_antisense	5'-cttgccgtaggtggcacagccgcagccctcgccggacacg-3'
K41C_T43C	5'-ggcgatgccacctacggctgcctgtgcctgaagttcatctgcac-3'
K41C_T43C_antisense	5'-gtgcagatgaacttcaggcacaggcagccgtaggtggcatcgcc-3'

EmGFP-3 (R109C, E111C, R122C, E124C)

Primer Name	Primer Sequence (5' to 3')
R109C_E111C	5'-cggcaactacaagacctgcgcctgcgtgaagttcgagggcg-3'
R109C_E111C_antisense	5'-cgccctcgaacttcacgcaggcgcaggtcttgtagttgccg-3'
R122C_E124C	5'-gcgacaccctggtgaactgcatctgcctgaagggcatcgactt-3'
R122C_E124C_antisense	5'-aagtcgatgcccttcaggcagatgcagttcaccagggtgtcgc-3'

EmGFP-4 (N149C, Y151C, Y200C, S202C)

Primer Name	Primer Sequence (5' to 3')
N149C_Y151C	5'-gtacaactacaacagccactgcgtctgtatcatggccgacaagcag-3'
N149C_Y151C_antisense	5'-ctgcttgtcggccatgatacagacgcagtggctgttgtagttgtac-3'
Y200C_S202C	5'-gctgcccgacaaccactgcctgtgcacccagt-3'
Y200C_S202C_antisense	5'-actgggtgcacaggcagtggttgtcgggcagc-3'

Colonies from the QuikChange Mutagenesis were mini-prepped (Qiagen) and sequenced by using the T7 Promoter sequencing primer (W.M. Keck Facility at Yale). Plasmids with the correct sequence were transformed into BL21 DE3 star One Shot cells (Invitrogen). Colonies were grown overnight in regular media (5 mL) supplemented with carbenicillin (starter culture). The next day, starter culture (0.5 mL) was added regular media (500 mL) supplemented with carbenicillin. When the cultures reached an OD_{600} of 0.4 (EmGFP-wt or EmGFP-2) or 0.3 (EmGFP-1), the cultures were induced with fresh Isopropyl β-D-1-thiogalactopyranoside (0.5 mM IPTG, Aldrich). The cultures were allowed to shake for 3 hours at 37^{o}C, afterwards the cells were immediately pelleted. BugBuster Protein Extractor Reagent (Novagen) supplemented with EDTA free complete protease inhibitor tablets (Roche) was added (5 mL per 1 g of pellet weight). The pellet was resuspended and incubated at room temperature for 20 min while shaking. The lysate was centrifuged for 20 min at 15 000 rpm. The supernantant was incubated with 4 mLs of washed Ni-NTA resin for 1 to 2 h and subsequently added to a PD-10 column. The resin was washed with 2 column volumes of wash buffer 1 (0.1 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP, 10 mM imidazole) and with 3 column volumes of wash buffer 2 (0.1 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP, 50 mM Imidazole). The protein was eluted with the elution buffer (0.1 M Tris HCl, 150 mM NaCl, 3.5 mM TCEP, 250 mM Imidazole). The samples were dialyzed into the binding buffer (0.1 M Tris HCl, 75 mM NaCl, 3.5 mM TCEP).

Circular dichroism (CD). Wavelength dependent CD spectra were acquired for various concentrations of wild-type p53, the p53 bipartite display mutants, wild-type EmGFP and the EmGFP bipartite display mutants at 25 ^{o}C in continuous scan mode with 0.5 nm data pitch, 50 nm/min scanning speed, 4 sec response, 0.5 nm band width and 3 accumulations using the Jasco J-810-150S spectropolarimeter.. Three trials were performed for each protein and the plots shown (Figure 2) are the average of these three trials with the background (buffer only) subtracted.

Measurement of EmGFP fluorescence. To determine if the fluorescence of the EmGFP-1 and EmGFP-2 was perturbed as a result of introducing four non-native cysteine residues, the fluorescence of EmGFP-wt, EmGFP-1 and EmGFP-2 as a function of concentration was determined (data not shown). The fluorescence was detected using an Analyst^{TM} AD plate reader (LJL Biosystems) with an excitation of 485 nm (Fluorescein channel) and emission of 530 nm (Fluorescein channel). No

significant difference in fluorescence was noticed between the wild-type and cysteine modified versions of EmGFP.

Determination of apparent equilibrium dissociation constants (K_d). Equilibrium dissociation constants of ReAsH•protein complexes were determined by monitoring the increase in ReAsH fluorescence intensity as a function of protein concentration. Titrations were performed in freshly prepared binding buffer (100 mM Tris-HCl, 75 mM NaCl, 3.5 mM TCEP, pH 7.8). In the case of the stringent buffer, it was supplemented with EDT (1 mM), while the permissive binding buffer was not supplemented with EDT. Fresh solutions of ReAsH-EDT_2 was prepared by dissolving a small amount (~0.1 mg) in DMSO and quantifying each stock solution (ReAsH ε_{578nm} = 63 000 cm^{-1} M^{-1}) upon dilution into 100 mM NaOH. The appropriate dilution of the ReAsH-EDT_2 stock solution was made into the fresh binding buffer to a concentration of 50 nM and was used immediately. The ReAsH (50 nM) buffer solution was mixed 1:1 with the appropriate dilution of protein and added to 384-well black, non-binding polystyrene Costar® plates (Corning Incorp.). These samples were sealed with Parafilm-M and incubated at room temperature without shaking. At the appropriate time point, the fluorescence emission λ_{630} (ReAsH) was detected using an Analyst^{TM} AD plate reader (LJL Biosystems). The changes in fluorescence emission as a function of protein concentration were plotted using Kaleidagraph v 3.6 (Synergy Software) and the apparent K_d values were fit to the equation below which is derived from first principals with no assumptions.

$$F_{obs} = F_{min} + ((F_{max} - F_{min})/(2[dye]))([dye] + [protein] + K_d - (([dye] + [protein] + K_d)^2 - 4[dye][protein])^{0.5})$$

In this equation, F_{obs} represents the observed fluorescence at any total protein concentration [protein], [dye] is the total concentration of ReAsH-EDT_2, and F_{max} and F_{min} are the maximum and minimum fluorescence values, respective. K_d is the apparent equilibrium dissociation constant. The reported K_d values represent the average ± standard deviation of the calculated K_d's of three or more independent trials.