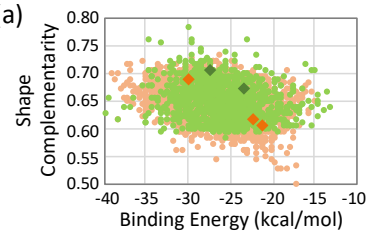
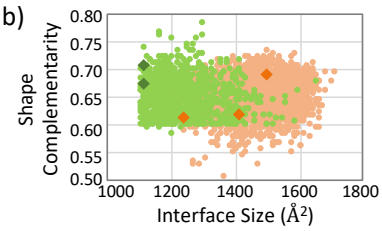
SUPPLEMENTARY MATERIALS

(a)



(b)

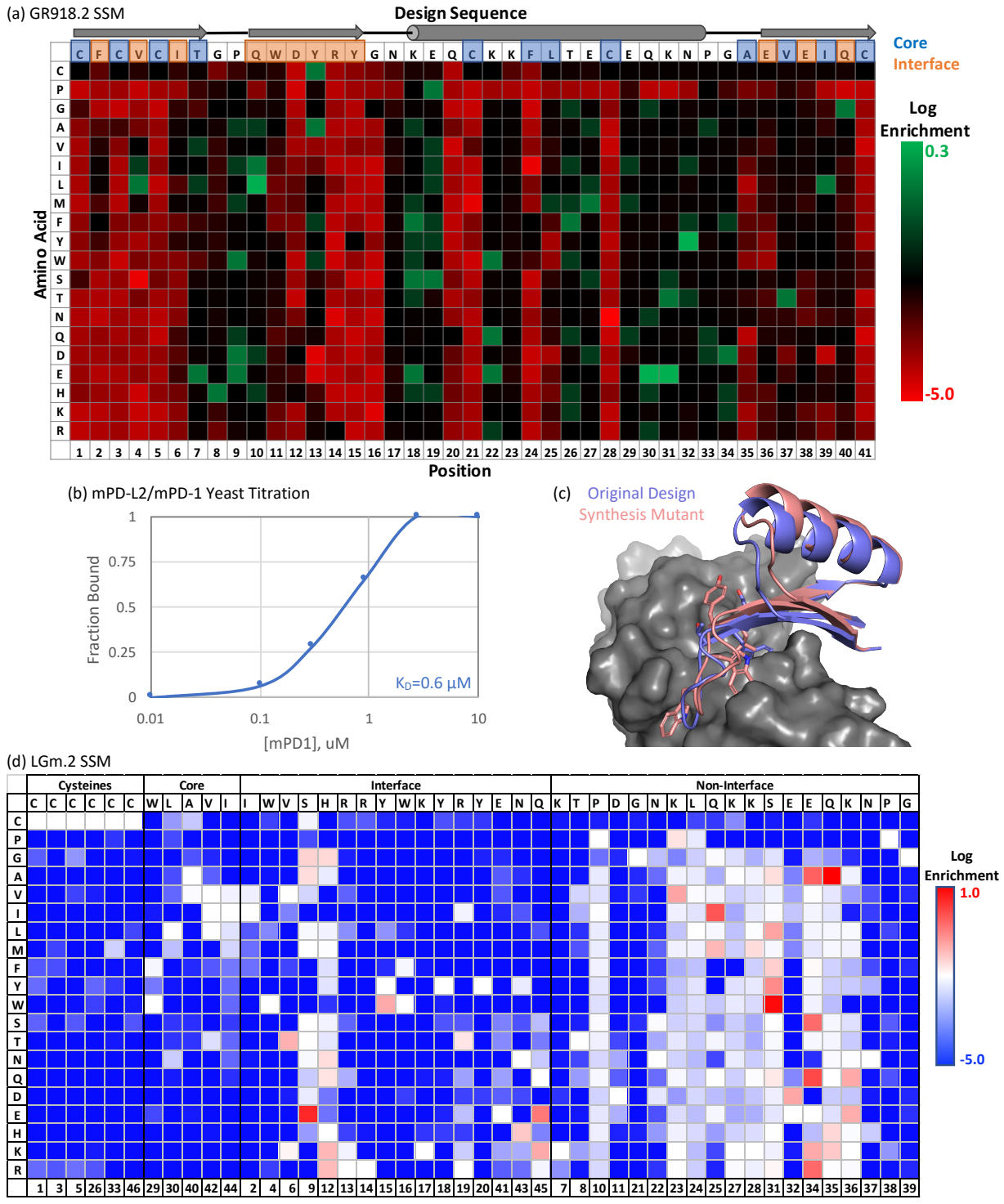


Motifgraft Designs
Fold-From-Loops Designs
Motifgraft Yeast Display Hits
Fold-From-Loops Yeast Display Hits

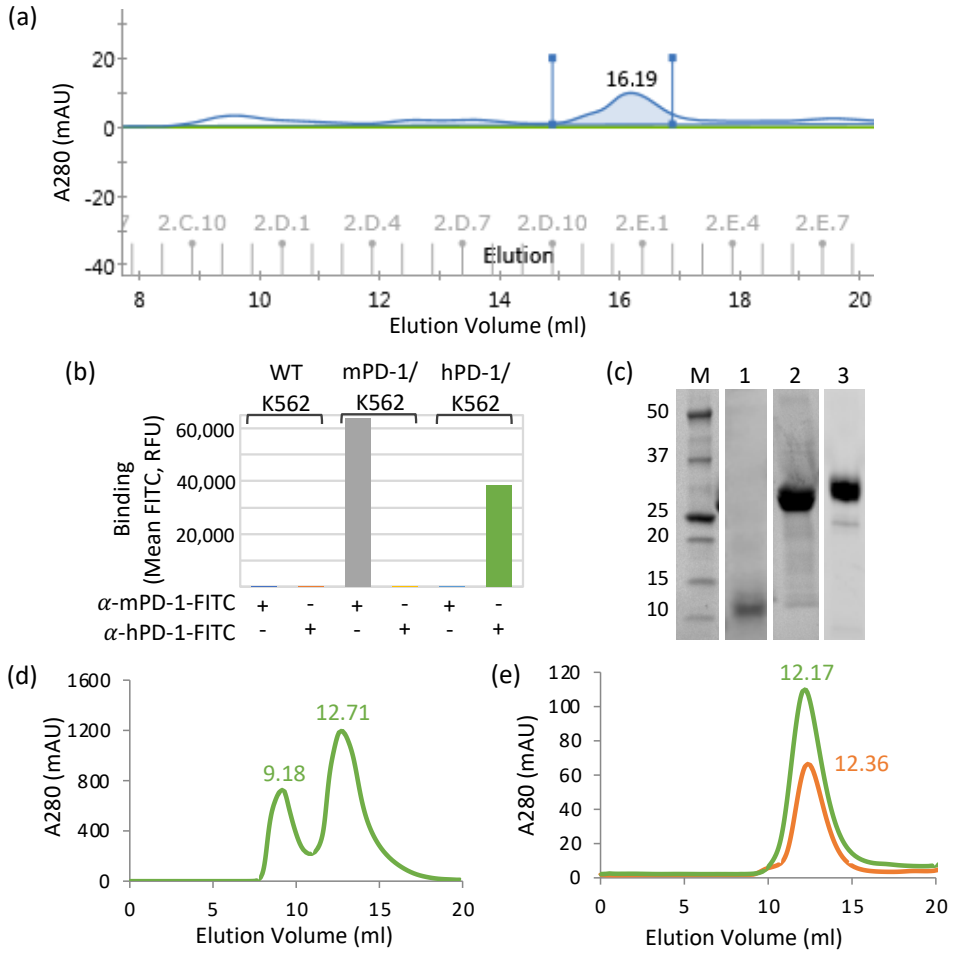
(c)

Design	Protocol	mPD-1 Enrichment	hPD-1 Enrichment	Fc Enrichment
GR918	Fold-From-Loops	786.5	0.3	0.3
GR829	Fold-From-Loops	98.1	0.0	0.0
CB23	Motifgraft	82.2	0.0	0.0
GR1945	Fold-From-Loops	31.2	0.0	0.1
CB1012	Motifgraft	0.0	461.8	0.0

Supplementary Figure 1: High-throughput screen of designed binders. (a) Shape complementarity and interface size of tested sequences. (b) Shape complementarity and binding energy distribution of tested sequences. (c) Hits in yeast display high-throughput screen.



Supplementary Figure 2: Additional affinity maturation figures. (a) Heat map representing the log enrichments for the GR918.2 SSM library selected with 2 µM mPD-1-Fc-biot. Enriched mutations are shown in green and depleted in red. (b) Titration of mPD-L2 displayed on yeast binding to mPD-1-Fc-biot with a Kd of 0.6 µM. (c) Rosetta model of the mutant found in the oligo array pool of loopgraft designs (pink) aligned to the original loopgraft design (purple) with the mutated residues shown in sticks. (d) Heat map representing the log enrichments for the LGm.2 SSM library selected with 2 µM hPD-1-Fc-biot. Enriched mutations are shown in red and depleted in blue.



Supplementary Figure 3: *In vitro* binding. (a) Gel filtration of GR918.2 monomer on a Superdex 75 Increase 10/300 GL column (GE Healthcare Life Sciences). (b) mPD-1/K562 and hPD-1/K562 stable cell lines were validated using anti-mPD-1-FITC and anti-hPD-1-FITC antibodies. (c) SDS-PAGE of various PD-MP1 constructs. M=Precision Plus Dual Xtra protein ladder (BioRad), 1=PD-MP1 monomer (9.7 kDa), 2=Spycatcher_2L6HC3_13 trimer (25.8 kDa), 3=PD-MP1_2L6HC3_13 trimer (35.5 kDa), (d) Gel filtration of PD-MP1 monomer on a Superdex 75 Increase 10/300 GL column (GE Healthcare Life Sciences). (e) Gel filtration of PD-MP1_2L6HC3_13 trimer (green) and Spycatcher_2L6HC3_13 unconjugated trimer (orange) on a Superdex 200 Increase 10/300 GL column (GE Healthcare Life Sciences).

(a) Step 1A: Backbone Grafting

```
<ROSETTASCRIPTS>
	<SCOREFXNS>
		<sfxn_std weights="talaris2013">
		</sfxn_std>
	</SCOREFXNS>
	<TASKOPERATIONS>
		<IncludeCurrent name=ic />
		<LimitAromaChi2 name=limitaro chi2max=110 chi2min=70 />
	</TASKOPERATIONS>
	<MOVERS>
		<MotifGraft name="motif_grafting" context_structure="./pd1_3bp5.pdb"
motif_structure="./motif_3bp5.pdb" RMSD_tolerance="0.7" NC_points_RMSD_tolerance="0.7"
clash_score_cutoff="10" clash_test_residue="ALA" combinatory_fragment_size_delta="0:0"
max_fragment_replacement_size_delta="0:0"  full_motif_bb_alignment="1"
allow_independent_alignment_per_fragment="0" graft_only_hotspots_by_replacement="0"
hotspots="1:3:5" revert_graft_to_native_sequence="1" allow_repeat_same_graft_output="0"/>
	</MOVERS>
	<APPLY_TO_POSE>
	</APPLY_TO_POSE>
	<PROTOCOLS resume_support=true>
		<Add mover_name="motif_grafting"/>
	</PROTOCOLS>
</ROSETTASCRIPTS>
```

(b) Step 1B: Sidechain Grafting

```
<ROSETTASCRIPTS>
	<SCOREFXNS>
		<sfxn_std weights="talaris2013">
		</sfxn_std>
	</SCOREFXNS>
	<TASKOPERATIONS>
		<IncludeCurrent name=ic />
		<LimitAromaChi2 name=limitaro chi2max=110 chi2min=70 />
	</TASKOPERATIONS>
	<MOVERS>
		<MotifGraft name="motif_grafting" context_structure="./pd1_3bp5.pdb"
motif_structure="./motif_3bp5.pdb" RMSD_tolerance="0.7" NC_points_RMSD_tolerance="0.7"
clash_score_cutoff="10" clash_test_residue="ALA" combinatory_fragment_size_delta="0:0"
max_fragment_replacement_size_delta="0:0"  full_motif_bb_alignment="1"
allow_independent_alignment_per_fragment="0" graft_only_hotspots_by_replacement="1"
hotspots="1:3:5" revert_graft_to_native_sequence="1" allow_repeat_same_graft_output="0"/>
	</MOVERS>
	<APPLY_TO_POSE>
	</APPLY_TO_POSE>
	<PROTOCOLS resume_support=true>
		<Add mover_name="motif_grafting"/>
	</PROTOCOLS>
</ROSETTASCRIPTS>
```

(c) Step 2: Sequence Design & Filtering

```
<ROSETTASCRIPTS>
	<SCOREFXNS>
		<sfxn_std weights="talaris2013">
		</sfxn_std>
		<sfxn_soft weights="soft_rep">
```

```
                </sfxn_soft>
                <talaris2013_cart weights="talaris2013">
                        <Reweight scoretype="cart_bonded" weight="1.0"/>
                        <Reweight scoretype="pro_close" weight="0.0"/>
                </talaris2013_cart>
                <talaris2013_cst weights="talaris2013">
                        <Reweight scoretype="coordinate_constraint" weight="1.0"/>
                </talaris2013_cst>
        </SCOREFXNS>
        <TASKOPERATIONS>
                <IncludeCurrent name=ic />
                <RestrictIdentities name=no_CYS identities=CYS prevent_repacking=1 />
                <DisallowIfNonnative name=dsgn_aa_core disallow_aas=CDEGHKMNQRST />
                <DisallowIfNonnative name=dsgn_aa_surf disallow_aas=ACFILMVWY />
                <ProteinInterfaceDesign name=PID repack_chain1=0 repack_chain2=1
design_chain1=0 design_chain2=1 interface_distance_cutoff=12/>
                <LimitAromaChi2 name=limitaro chi2max=110 chi2min=70 />
                <SelectBySASA name=core mode="sc" state="bound" core_asa=20 surface_asa=0
core=1 boundary=0 surface=0 verbose=1 />
                <SelectBySASA name=surf mode="sc" state="bound" core_asa=0 surface_asa=40
core=0 boundary=0 surface=1 verbose=1 />
                <OperateOnCertainResidues name=cst_graft>
                        <PreventRepackingRLT/>
                        <ResiduePDBInfoHasLabel property="HOTSPOT"/>
                </OperateOnCertainResidues>
        </TASKOPERATIONS>
        <FILTERS>
                <Geometry name="omega" omega="165" cart_bonded="20" start="115"
confidence="1"/>
                <EnergyPerResidue name="energy_per_res_filter" scorefxn="talaris2013"
score_type="total_score" energy_cutoff="3.0"  whole_interface="1" jump_number="1"
interface_distance_cutoff="8.0" bb_bb="0" confidence="0"/>
                <ShapeComplementarity name=sc_filt jump=1 verbose=1 min_sc=0.65
write_int_area=0 confidence=1 />
                <Ddg name=ddG_filt scorefxn=talaris2013 jump=1 repack=1 repeats=3 threshold=-10
confidence=1 />
                <BuriedUnsatHbonds name=buriedUnsatBonds scorefxn=talaris2013 jump_number=1
cutoff=9 confidence=0/>
                <Sasa name=sasa_int_area threshold=1100 upper_threshold=10000 hydrophobic=0
polar=0 jump=1 confidence=1 />
                <ScoreType name=total_score scorefxn=talaris2013 score_type=total_score
threshold=0 confidence=0/>
                <FragmentLookupFilter name="faulty_fragments"
lookup_name="source_fragments_4_mer"
store_path="/lab/databases/VALL_clustered/backbone_profiler_database_06032014"
lookup_mode="first" chain="2" threshold="0"  confidence="1" />
        </FILTERS>
        <MOVERS>
                <AddConstraintsToCurrentConformationMover name=add_heavy_coor_epitope_cst
use_distance_cst=0 coord_dev=0.05 bound_width=0.01 min_seq_sep=8  max_distance=12.0
cst_weight=1.0 task_operations=cst_graft CA_only=0 bb_only=1/>
                <PackRotamersMover name=design_soft scorefxn=talaris2013
task_operations=ic,no_CYS,PID,limitaro,cst_graft,dsgn_aa_core,core />
                <PackRotamersMover name=design_hard scorefxn=talaris2013
task_operations=ic,no_CYS,PID,limitaro,cst_graft,dsgn_aa_core,core />
```

```
		<PackRotamersMover name=design_surf scorefxn=talaris2013
task_operations=ic,no_CYS,PID,limitaro,cst_graft,dsgn_aa_surf,surf />
		<TaskAwareMinMover name=min scorefxn=talaris2013 bb=1 chi=1 jump=1
cartesian=0 task_operations=PID />
		<TaskAwareMinMover name=cart_min  scorefxn=talaris2013_cart  bb=1 chi=1 jump=1
cartesian=1 task_operations=PID /> #allows minimization of distances, not just bond angles
	</MOVERS>
	<APPLY_TO_POSE>
	</APPLY_TO_POSE>
	<PROTOCOLS resume_support=true>
		<Add mover_name=add_heavy_coor_epitope_cst />
		<Add mover_name=cart_min />
		<Add filter_name=omega />
		<Add mover_name=design_soft />
		<Add mover_name=min />
		<Add mover_name=design_hard />
		<Add mover_name=min />
		<Add mover_name=design_surf />
		<Add mover_name=cart_min />
		<Add filter_name=energy_per_res_filter />
		<Add filter_name=faulty_fragments/>
		<Add filter_name=sc_filt />
		<Add filter_name=ddG_filt />
		<Add filter_name=buriedUnsatBonds />
		<Add filter_name=sasa_int_area />
		<Add filter_name=total_score />
	</PROTOCOLS>
</ROSETTASCRIPTS>
```

Supplementary Figure 4: Motifgraft Scripts. (a) RosettaScripts xml file using the backbone grafting version of Motifgraft. (b) RosettaScripts xml file using the sidechain grafting version of Motifgraft. (c) RosettaScripts xml for sequence design and filtering of motifgrafts.

(a) Step 1: *De Novo* Backbone Generation & Addition of Disulfide Bonds

```
<dock_design>
        <SCOREFXNS>
                <sfxn_std  weights=talaris2013/>
                <SFXN1 weights="fldsgn_cen">
                        <Reweight scoretype="hbond_sr_bb" weight="1.0" />
                        <Reweight scoretype="hbond_lr_bb" weight="1.0" />
                        <Reweight scoretype="atom_pair_constraint" weight="1.0" />
                        <Reweight scoretype="angle_constraint" weight="1.0" />
                        <Reweight scoretype="dihedral_constraint" weight="1.0" />
                </SFXN1>
                <SFXN2 weights="fldsgn_cen">
                        <Reweight scoretype="hbond_sr_bb" weight="1.0" />
                        <Reweight scoretype="hbond_lr_bb" weight="1.0" />
                        <Reweight scoretype="atom_pair_constraint" weight="1.0" />
                        <Reweight scoretype="angle_constraint" weight="1.0" />
                        <Reweight scoretype="dihedral_constraint" weight="1.0" />
                </SFXN2>
        </SCOREFXNS>
        <FILTERS>
                <ScoreType name="hbond_sfn" scorefxn=sfxn_std score_type=hbond_lr_bb
threshold=0/>
                <ResidueCount name="cys_count" residue_types="CYD" confidence="0" />
                <ResidueCount name="res_count" confidence="0" />
                <ResidueCount name="cyd4" residue_types="CYD" confidence="1"
min_residue_count=4 max_residue_count=4/>
                <DisulfideEntropy name="entropy12" lower_bound=11.8 tightness=1000 />
                <CompoundStatement name="2ds" >
                        <AND filter_name=entropy12 />
                        <AND filter_name=cyd4 />
                </CompoundStatement>
                <ResidueCount name="cyd6" residue_types="CYD" confidence="1"
min_residue_count=6 max_residue_count=6/>
                <DisulfideEntropy name="entropy16" lower_bound=16 tightness=1000 />
                <CompoundStatement name="3ds" >
                        <AND filter_name=entropy16 />
                        <AND filter_name=cyd6 />
                </CompoundStatement>
                <ResidueCount name="4ds" residue_types="CYD" confidence="1"
min_residue_count=8 max_residue_count=8/>
                <CompoundStatement name="ds_entropy" >
                        <OR filter_name=2ds />
                        <OR filter_name=3ds />
                        <OR filter_name=4ds />
                </CompoundStatement>
                <CalculatorFilter name=bb equation="hbond / rescount" threshold="-0.22"
confidence=1>
                        <VAR name="hbond" filter="hbond_sfn"/>
                        <VAR name="rescount" filter="res_count"/>
                </CalculatorFilter>
                <ScoreType name="dslf_fa13" scorefxn=sfxn_std score_type=dslf_fa13 threshold=0/>
                <CalculatorFilter name=mean_dslf equation="dslf / cyscount" threshold="-0.30"
confidence=1>
                        <VAR name="dslf" filter="dslf_fa13"/>
                        <VAR name="cyscount" filter="cys_count"/>
                </CalculatorFilter>
```

```
                <SheetTopology name="sf1" blueprint="blueprint" />
                <SecondaryStructure name="ss1" blueprint="blueprint.ss" />
                <CompoundStatement name="cs1">
                        <AND filter_name="ss1" />
                        <AND filter_name="sf1" />
                </CompoundStatement>
                <ScoreType name="total_score_cen" score_type="total_score" scorefxn="SFXN2"
confidence="0" threshold="0" />
                <AverageDegree name=degree confidence=1 threshold=9.5/>
                <DisulfideEntropy name="entropy" lower_bound=11.5 tightness=1000 />
                <PackStat name=pack confidence=0/>
                <ExposedHydrophobics name=exposed confidence=0/>
                <AtomicContactCount name=contact confidence=0/>
                <CavityVolume name=cavity confidence=0/>
        </FILTERS>
        <TASKOPERATIONS>
                <LimitAromaChi2 name="limitchi2" include_trp="1" />
                <LayerDesign name="layer_all" layer="core_boundary_surface_Nterm_Cterm"
use_sidechain_neighbors=True pore_radius="2.0" verbose="true" />
                <NoRepackDisulfides name="exemptdisulf" />
                <OperateOnCertainResidues name="keep_pd1">
                        <PreventRepackingRLT/>
                        <ResidueIndexIs indices=11,13,15/>
                </OperateOnCertainResidues>
        </TASKOPERATIONS>
        <MOVERS>
                <Dssp name="dssp" />
                <SheetCstGenerator name="sheet_new1" cacb_dihedral_tolerance="0.6"
blueprint="blueprint" />
                <RemoveCsts name="sheet_rm1" generator="sheet_new1" />
                <SetSecStructEnergies name="set_ssene1" scorefxn="SFXN1" blueprint="blueprint"
/>
                <BluePrintBDR name="bdr1" use_abego_bias="1" scorefxn="SFXN1"
constraint_generators="sheet_new1" constraints_NtoC="1.0" blueprint="blueprint" />
                <DumpPdb name="dump" fname="success" tag_time=True/>
                <RemodelMover name="remodel" fast_disulf="True" match_rt_limit="2"
quick_and_dirty="True" bypass_fragments="True" min_disulfides=1 max_disulfides=3 min_loop=8/>
                <FastDesign name="fastdes" task_operations="limitchi2,layer_all,keep_pd1"
scorefxn="sfxn_std" allow_design="1" clear_designable_residues="0" repeats="2"
ramp_down_constraints="0" >
                        <MoveMap name="mm">
                                <Span begin=11 end=15 chi=True  bb=False />
                                <Span begin=11 end=11 chi=False bb=False />
                                <Span begin=13 end=13 chi=False bb=False />
                                <Span begin=15 end=15 chi=False bb=False />
                        </MoveMap>
                </FastDesign>
                <FastDesign name="fastdes7" task_operations="limitchi2,layer_all,keep_pd1"
scorefxn="sfxn_std" allow_design="1" clear_designable_residues="0" repeats="5"
ramp_down_constraints="0" >
                        <MoveMap name="mm">
                                <Span begin=11 end=15 chi=True  bb=False/>
                                <Span begin=11 end=11 chi=False bb=False />
                                <Span begin=13 end=13 chi=False bb=False />
                                <Span begin=15 end=15 chi=False bb=False />
                        </MoveMap>
```

```
                </FastDesign>
                <ParsedProtocol name="build_disulf">
                        <Add mover_name="remodel" />
                        <Add mover_name="fastdes"/>
                        <Add mover_name="remodel" />
                        <Add mover_name="fastdes"/>
                        <Add mover_name="remodel" />
                        <Add filter_name="entropy"/>
                        <Add mover_name="fastdes7"/>
                        <Add filter_name="degree" />
                        <Add filter_name="bb" />
                        <Add filter_name="mean_dslf" />
                        <Add mover_name="dump"/>
                </ParsedProtocol>
                <LoopOver name="disulfide_loop" mover_name="build_disulf" iterations="20" drift="0"
ms_whenfail="FAIL_DO_NOT_RETRY" />
                <ParsedProtocol name="build_dssp1" >
                        <Add mover_name="bdr1" />
                        <Add mover_name="dssp" />
                        <Add filter_name="cs1" />
                        <Add filter_name="degree" />
                        <Add mover_name="disulfide_loop" />
                </ParsedProtocol>
                <LoopOver name="lover1" mover_name="build_dssp1" iterations="1000" drift="0"
ms_whenfail="FAIL_DO_NOT_RETRY" />
                <ParsedProtocol name="phase1" >
                        <Add mover_name="set_ssene1" />
                        <Add mover_name="lover1" />
                </ParsedProtocol>
        </MOVERS>
        <PROTOCOLS>
                <Add mover_name="phase1" />
                <Add mover_name="dssp" />
                <Add filter_name="total_score_cen" />
                <Add filter_name="cs1" />
        </PROTOCOLS>
</dock_design>
```

blueprint

```
SSPAIR 1-2.A.0;1-3.A.0
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   LX   R
0   V   LX   R
0   V   EB   R
0   V   EB   R
4   W   EB   .
5   V   EB   .
6   Y   EB   .
7   V   EB   .
8   Y   EB   .
0   V   LX   R
```

blueprint.ss

```
0   V   D   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   D   R
0   V   LX   R
0   V   LX   R
0   V   D   R
0   V   EB   R
4   W   D   .
5   V   D   .
6   Y   D   .
7   V   D   .
8   Y   D   .
0   V   LX   R
0   V   LX   R
```

```
0   V   LX   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   HA   R
0   V   LX   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
```

```
0   V   D   R
0   V   D   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   H   R
0   V   D   R
0   V   D   R
0   V   LX   R
0   V   D   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   EB   R
0   V   D   R
```

(b) Step 2: Sequence Design in Presence of PD-1 Target

```
<dock_design>
    <SCOREFXNS>
        <sfxn_std  weights=talaris2013>
            <Reweight scoretype="dslf_fa13" weight="2.0" />
        </sfxn_std >
        <SFXN1 weights="fldsgn_cen">
            <Reweight scoretype="hbond_sr_bb" weight="1.0" />
            <Reweight scoretype="hbond_lr_bb" weight="1.0" />
            <Reweight scoretype="atom_pair_constraint" weight="1.0" />
            <Reweight scoretype="angle_constraint" weight="1.0" />
            <Reweight scoretype="dihedral_constraint" weight="1.0" />
        </SFXN1>
        <SFXN2 weights="fldsgn_cen">
            <Reweight scoretype="hbond_sr_bb" weight="1.0" />
            <Reweight scoretype="hbond_lr_bb" weight="1.0" />
            <Reweight scoretype="atom_pair_constraint" weight="1.0" />
            <Reweight scoretype="angle_constraint" weight="1.0" />
            <Reweight scoretype="dihedral_constraint" weight="1.0" />
        </SFXN2>
        <sfxn_soft weights=soft_rep />
        <clash weights=empty symmetric=0>
        <Reweight scoretype=fa_atr weight=0.8/>
            <Reweight scoretype=fa_rep weight=0.44/>
        </clash>
        <CysCounter
weights="/work/grocklin/miniproteins/EEHEE/bigger1/bps/cys_counter_weights.wts"/>
        <ResCounter
weights="/work/grocklin/miniproteins/EEHEE/bigger1/bps/res_counter_weights.wts"/>
    </SCOREFXNS>
    <FILTERS>
        <ScoreType name="cys_count" scorefxn=CysCounter threshold=200/>
```

```
        <ScoreType name="res_count" scorefxn=ResCounter threshold=200/>
        <ScoreType name="dslf_fa13" scorefxn=sfxn_std score_type=dslf_fa13 threshold=0/>
        <ScoreType name="hbond_sfn" scorefxn=sfxn_std score_type=hbond_lr_bb threshold=0/>
        <CalculatorFilter name=cc equation="- cyscount" threshold="-3" confidence=1>
            <VAR name="cyscount" filter="cys_count"/>
        </CalculatorFilter>
        <CalculatorFilter name=bb equation="hbond / rescount" threshold="-0.25" confidence=1>
            <VAR name="hbond" filter="hbond_sfn"/>
            <VAR name="rescount" filter="res_count"/>
        </CalculatorFilter>
        <CalculatorFilter name=mean_dslf equation="dslf / cyscount" threshold="-0.35" confidence=1>
            <VAR name="dslf" filter="dslf_fa13"/>
            <VAR name="cyscount" filter="cys_count"/>
        </CalculatorFilter>
        <Ddg name=clash  scorefxn=clash threshold=2 jump=1 repeats=1 repack=0 confidence=1/>
        <Ddg name=ddg  scorefxn=sfxn_std threshold=-10 jump=1 repeats=1 repack=0
confidence=1/>
        <Rmsd name=rmsdToPeptide threshold=0.45 superimpose=0>   0.45
        <span_two begin_native=4 end_native=8 begin_pose=11 end_pose=15 CA_only=0/>
        </Rmsd>
        <CompoundStatement name="ddg_clashes">
            <AND filter_name="clash" />
            <AND filter_name="ddg" />
            <AND filter_name="rmsdToPeptide" />
        </CompoundStatement>
        <CompoundStatement name="monomer">
            <AND filter_name="bb" />
            <AND filter_name="mean_dslf" />
        </CompoundStatement>
        <Holes name="holes" threshold="6.0" confidence="0" />
        <ScoreType name="total_score_cen" score_type="total_score" scorefxn="SFXN2"
confidence="0" threshold="0" />
    </FILTERS>
    <TASKOPERATIONS>
        <LimitAromaChi2 name="limitchi2" include_trp="1" />
        <LayerDesign name="layer_all" layer="Nterm_core_boundary_surface_Cterm"
use_sidechain_neighbors=True pore_radius="2.0" verbose="true" />
        <NoRepackDisulfides name="exemptdisulf" />
        <OperateOnCertainResidues name="keepron2">
            <PreventRepackingRLT/>
            <ResidueIndexIs indices=11,13,15/>
        </OperateOnCertainResidues>
        <OperateOnCertainResidues name=chainA>
            <PreventRepackingRLT/>
            <ChainIs chain=B/>
        </OperateOnCertainResidues>
    </TASKOPERATIONS>
    <MOVERS>
        <Dssp name="dssp" />
        <DumpPdb name="Dump1" fname="success" tag_time=True />
        <Superimpose name=alignToPeptide ref_start=4 ref_end=8  target_start=11 target_end=15
CA_only=0/>
        <Superimpose name=alignToTarget ref_start=10 ref_end=123
target_start=%%target_start%% target_end=%%target_end%% CA_only=1/>
        <SwitchChainOrder name="cutout" chain_order="1"/>
```

```
        <AddChain name=addTarget
file_name="/work/grocklin/cassie/protG_with_motif/design_align/pd1.pdb" new_chain=1
scorefxn=sfxn_std random_access=0/>
        <FastDesign name="fastdes" task_operations="limitchi2,layer_all,keepron2,chainA"
scorefxn="sfxn_std" allow_design="1" clear_designable_residues="0" repeats="1"
ramp_down_constraints="0" >
            <MoveMap name="mm">
                <Chain number=2 chi=False bb=False/>
        </MoveMap>
        </FastDesign>
        <ParsedProtocol name="design_align" >
            <Add mover_name="fastdes" />
            <Add mover_name="alignToPeptide" />
            <Add mover_name="cutout" />
            <Add mover_name="addTarget" />
        </ParsedProtocol>
        <LoopOver name="3xdes" mover_name="design_align" iterations="5" drift="1"
filter_name=true_filter />
        <ParsedProtocol name="3xdes_des" >
            <Add mover_name="3xdes" />
            <Add mover_name="fastdes" />
            <Add mover_name="alignToTarget" />
            <Add filter_name="ddg_clashes" />
            <Add mover_name="cutout" />
            <Add filter_name="monomer" />
            <Add mover_name="addTarget" />
            <Add mover_name="Dump1" />
        </ParsedProtocol>
        <LoopOver name="lover2" mover_name="3xdes_des" iterations="10" drift="0"
ms_whenfail="FAIL_DO_NOT_RETRY" />
    </MOVERS>
    <PROTOCOLS>
        <Add mover_name="lover2" />
        <Add mover_name="dssp" />
    </PROTOCOLS>
</dock_design>
```

(c) Step 3: Optimize Sequence

```
<dock_design>
    <SCOREFXNS>
        <SFXN7 weights="talaris2013.wts">
            <Reweight scoretype=res_type_constraint weight=1/>
        </SFXN7>
    </SCOREFXNS>
    <TASKOPERATIONS>
        <LimitAromaChi2 name="limitchi2" />
        <LayerDesign name="layer" layer="core_boundary_surface_Nterm_Cterm" pore_radius="2.0"
verbose="true" use_sidechain_neighbors=True/>
    <LayerDesign name="layer_surface" layer="surface" pore_radius="2.0" verbose="true"
use_sidechain_neighbors=True/>
        <RestrictToRepacking name=restrict />
        <DesignByResidueCentrality name="design_central_residues" regions_to_design="5"
region_shell="0" repack_non_selected="0" />
        <OperateOnCertainResidues name="keepron2">
            <PreventRepackingRLT/>   all plus 297
            <ResidueIndexIs indices=11,13,15/>
```

```
        </OperateOnCertainResidues>
        <OperateOnCertainResidues name=chainA>
            <PreventRepackingRLT/>
            <ChainIs chain=B/>
        </OperateOnCertainResidues>
        <NoRepackDisulfides name="exemptdisulf" />
        <ReadResfile name="rrf" filename="resfile" />
    </TASKOPERATIONS>
    <MOVERS>
        <FastRelax name="relax_test" repeats="2" scorefxn="SFXN7"
task_operations="limitchi2,keepron2,chainA,restrict">
            <MoveMap name="mm">
                <Chain number=2 chi=False bb=False/>
        </MoveMap>
        </FastRelax>
        <FastRelax name="full_relax" scorefxn="SFXN7"
task_operations="limitchi2,keepron2,chainA,restrict">
            <MoveMap name="mm">
                <Chain number=2 chi=False bb=False/>
        </MoveMap>
        </FastRelax>
        <TaskAwareMinMover name="bb_min" scorefxn="SFXN7" bb="1" chi="1" jump="1"
task_operations="limitchi2,keepron2,chainA,rrf" tolerance="0.0001" />
        <PackRotamersMover name="design_hard" scorefxn="SFXN7"
task_operations="limitchi2,keepron2,chainA,rrf" />
        <ParsedProtocol name="design_min" >
            <Add mover_name="design_hard" />
            <Add mover_name="bb_min" />
        </ParsedProtocol>
    </MOVERS>
    <FILTERS>
        <ResidueCount name="nres_cys" residue_types="CYD" confidence="0" />
        <ResidueCount name="nres" confidence="0" />
        <ScoreType name="dslf_fa13" scorefxn=SFXN7 score_type=dslf_fa13 threshold=0/>
        <ScoreType name="hbond_sfn" scorefxn=SFXN7 score_type=hbond_lr_bb threshold=0/>
        <SSPrediction name="psipred_prob" confidence="0"
cmd="/gscratch/baker/tlinsky/source/psipred/runpsipred_single" use_probability="1"
mismatch_probability=True use_svm="0" />
        <SSShapeComplementarity name="ss_sc" verbose="0" confidence="0" />
        <TotalSasa name="exposed_hydrophobics" confidence="0" hydrophobic=True />
        <CalculatorFilter name=bb equation="hbond / rescount" threshold="-0.23" confidence=0>
        <VAR name="hbond" filter="hbond_sfn"/>
        <VAR name="rescount" filter="nres"/>
        </CalculatorFilter>
        <CalculatorFilter name=mean_dslf equation="(dslf + 0.937) / (cyscount-2)" threshold="-0.35"
confidence=0>
            <VAR name="dslf" filter="dslf_fa13"/>
            <VAR name="cyscount" filter="nres_cys"/>
        </CalculatorFilter>
        <RotamerBoltzmannWeight name="rotamer_boltz_core_avg"
task_operations="design_central_residues" skip_ala_scan="1" scorefxn="SFXN7"
no_modified_ddG="1" />
        <CavityVolume name="cavity_volume" />
        <PackStat name=pack confidence=0/>
        <Holes name="holes" confidence=0/>
        <Ddg name=ddg  scorefxn=SFXN7 threshold=-10 jump=1 repeats=1 repack=0 confidence=0/>
```

```
        <Rmsd name=rmsdToPeptide threshold=0.45 superimpose=0>   0.45
        <span_two begin_native=4 end_native=8 begin_pose=11 end_pose=15 CA_only=0/>
        </Rmsd>
    </FILTERS>
    <MOVERS>
        <Dssp name="dssp" />
        <DumpPdb name="AfterSuper" fname="AfterSuper.pdb" tag_time=True />
        <DumpPdb name="AfterRelax" fname="AfterRelax.pdb" tag_time=True />
        <DumpPdb name="dump" fname="h.pdb" tag_time=True />
        <Superimpose name=super_target ref_start=10 ref_end=123  target_start=%%target_start%%
target_end=%%target_end%% CA_only=1/>
        <FastDesign name="fastdes" scorefxn="SFXN7" repeats="3"
task_operations="limitchi2,keepron2,chainA,layer_surface">
            <MoveMap name="mm">
                <Chain number=2 chi=False bb=False/>
        </MoveMap>
        </FastDesign>
        <FastRelax name="relax_for_greedy" scorefxn="SFXN7" repeats="1"
task_operations="limitchi2,keepron2,chainA,restrict">
            <MoveMap name="mm">
                <Chain number=2 chi=False bb=False/>
        </MoveMap>
        </FastRelax>
        <ParsedProtocol name="relax_for_greedy_fix">
            <Add mover_name="relax_for_greedy" />
            <Add mover_name="super_target" />
        </ParsedProtocol>
        <RestrictRegion name="restrict_worst_psipred" type="psipred" scorefxn="SFXN7"
psipred_cmd="/gscratch/baker/tlinsky/source/psipred/runpsipred_single" max_trp="1"
task_operations="limitchi2,layer,keepron2,chainA"  resfile="resfile" />
        <RestrictRegion name="restrict_worst_exposed" type="packstat" scorefxn="SFXN7"
psipred_cmd="/gscratch/baker/tlinsky/source/psipred/runpsipred_single" max_trp="1"
task_operations="limitchi2,layer,keepron2,chainA"  resfile="resfile" />
        <RestrictRegion name="restrict_worst_random_mut" type="random_mutation"
scorefxn="SFXN7" psipred_cmd="/gscratch/baker/tlinsky/source/psipred/runpsipred_single"
max_trp="1" task_operations="limitchi2,layer,keepron2,chainA"  resfile="resfile" />
        <RestrictRegion name="restrict_worst_score" type="score" scorefxn="SFXN7"
psipred_cmd="/gscratch/baker/tlinsky/source/psipred/runpsipred_single" max_trp="1"
task_operations="limitchi2,layer,keepron2,chainA"  resfile="resfile" />
        <ParsedProtocol name="restrict_worst_random" mode="single_random">
            <Add mover_name="restrict_worst_psipred" />
            <Add mover_name="restrict_worst_random_mut" />
            <Add mover_name="restrict_worst_score" />
        </ParsedProtocol>
        <GenericMonteCarlo name="repeat_refinement" mover_name="design_min"
scorefxn_name="SFXN7" recover_low="1" trials="5" sample_type="low" preapply="0" drift="1"
temperature="0"/>
        <ParsedProtocol name="mutate_residue" >
            <Add mover_name="restrict_worst_random" />
            <Add mover_name="repeat_refinement" />
            <Add mover_name="super_target" />
        </ParsedProtocol>
        <GenericSimulatedAnnealer name="optimize_pose" mover_name="mutate_residue"
trials="15000" periodic_mover="relax_for_greedy_fix" eval_period="40" history="40" bolz_rank="1"
recover_low="1" preapply="0" drift="1" checkpoint_file="mc" keep_checkpoint_file="1"
filter_name="psipred_prob" temperature="0.0002" sample_type="low" >
```

```
            <Filters>
            <AND filter_name="cavity_volume" temperature="5.0" sample_type="low"/>
            <AND filter_name="pack" temperature="0.07" sample_type="high" />
            <AND filter_name="bb" temperature="0.001" sample_type="low" />
            <AND filter_name="mean_dslf" temperature="0.005" sample_type="low" />
            <AND filter_name="ddg" temperature="1" sample_type="low"/>
            <AND filter_name="rmsdToPeptide" temperature=0.001 sample_type="low" />
            </Filters>
        </GenericSimulatedAnnealer>
    </MOVERS>
    <PROTOCOLS>
        <Add mover_name="fastdes" />
        <Add mover_name="dssp" />
        <Add mover_name="optimize_pose" />
        <Add mover_name="full_relax" />
        <Add filter_name="psipred_prob" />
        <Add filter_name="cavity_volume" />
        <Add filter_name="ss_sc" />
        <Add filter_name="holes" />
        <Add filter_name="bb" />
        <Add filter_name="mean_dslf" />
    </PROTOCOLS>
</dock_design>
```

(d) Step 4: Filtering

```
<dock_design>
    <SCOREFXNS>
        <SFXN7 weights="talaris2013.wts" >
        </SFXN7>
        <TotalHydrophobic
weights="/work/grocklin/miniproteins/EEHEE/bigger1/bps/total_hydrophobic_weights.wts"/>
    </SCOREFXNS>
    <TASKOPERATIONS>
        <LayerDesign name="layer_core_SCN" layer="core" pore_radius="2.0" verbose="true"
use_sidechain_neighbors="True" />
        <LayerDesign name="layer_core_SASA" layer="core" core="20" pore_radius="2.0"
verbose="true" />
        <NoRepackDisulfides name="exemptdisulf" />
        <OperateOnCertainResidues name="no_ala_disulf" >
        <RestrictToRepackingRLT />
        <ResidueName3Is name3="ALA,CYS,CYD" />
        </OperateOnCertainResidues>
    </TASKOPERATIONS>
    <FILTERS>
        <AverageDegree name="degree_core_SCN" task_operations="layer_core_SCN"
confidence="0" threshold="9.4" />
        <AverageDegree name="degree_core_SASA" task_operations="layer_core_SASA"
confidence="0" threshold="9.4" />
        <AverageDegree name=degree confidence=0 threshold=9.5/>
        <ResidueCount name="res_count_all" max_residue_count="9999" confidence="0"/>
        <ResidueCount name="res_count_core_SCN"
task_operations="layer_core_SCN,no_ala_disulf" max_residue_count="9999" confidence="0"/>
        <ResidueCount name="res_count_core_SASA"
task_operations="layer_core_SASA,no_ala_disulf" max_residue_count="9999" confidence="0"/>
        <ResidueCount name="res_count_CYS_CYD" residue_types="CYS,CYD"
max_residue_count="9999" confidence="0"/>
```

```
        <ResidueCount name="res_count_CYD" residue_types="CYD" max_residue_count="9999"
confidence="0"/>
        <ScoreType name="dslffa13"  score_type="dslf_fa13" threshold="0" confidence="0"/>
        <ResidueCount name="cyd4" residue_types="CYS,CYD" confidence="1"
min_residue_count=4 max_residue_count=4/>
        <DisulfideEntropy name="entropy12" lower_bound=11.8 tightness=1000 />
        <CompoundStatement name="2ds" >
            <AND filter_name=entropy12 />
            <AND filter_name=cyd4 />
        </CompoundStatement>
        <TotalSasa name="total_sasa" threshold="1" upper_threshold="1000000000000000"
report_per_residue_sasa="False" confidence="0" />
        <CalculatorFilter name="mean_sasa" equation="Tsasa / rescount2" threshold="-0.30"
confidence="0" >
            <VAR name="Tsasa" filter="total_sasa"/>
            <VAR name="rescount2" filter="res_count_all"/>
        </CalculatorFilter>
        <CalculatorFilter name="percent_core_SCN" equation="rescount_coreSCN / rescount3"
threshold="-0.35" confidence="0" >
            <VAR name="rescount3" filter="res_count_all"/>
            <VAR name="rescount_coreSCN" filter="res_count_core_SCN"/>
        </CalculatorFilter>
        <CalculatorFilter name="percent_core_SASA" equation="rescount_coreSASA / rescount4"
threshold="-0.35" confidence="0" >
            <VAR name="rescount4" filter="res_count_all"/>
            <VAR name="rescount_coreSASA" filter="res_count_core_SASA"/>
        </CalculatorFilter>
        <AtomicContactCount name="contact_all" confidence="0" />
        <AtomicContactCount name="contact_core_SCN" task_operations="layer_core_SCN"
confidence="0" />
        <AtomicContactCount name="contact_core_SASA" task_operations="layer_core_SASA"
confidence="0" />
        <ResidueCount name="cyd6" residue_types="CYD,CYS" confidence="1"
min_residue_count=6 max_residue_count=6/>
        <DisulfideEntropy name="entropy16" lower_bound=16 tightness=1000 />
        <CompoundStatement name="3ds" >
            <AND filter_name=entropy16 />
            <AND filter_name=cyd6 />
        </CompoundStatement>
        <ResidueCount name="4ds" residue_types="CYD,CYS" confidence="1" min_residue_count=8
max_residue_count=8/>
        <CompoundStatement name="ds_entropy" >
            <OR filter_name=2ds />
            <OR filter_name=3ds />
            <OR filter_name=4ds />
        </CompoundStatement>
        <AveragePathLength name="apl" confidence="0" />
        <DisulfideEntropy name="entropy" confidence="0" />
        <ResidueCount name="nres_cys" residue_types="CYD" confidence="0" />
        <ResidueCount name="nres" confidence="0" />
        <ScoreType name="dslf_fa13" scorefxn=SFXN7 score_type=dslf_fa13 threshold=0/>
        <ScoreType name="hbond_sfn" scorefxn=SFXN7 score_type=hbond_lr_bb threshold=0/>
        <SSShapeComplementarity name="ss_sc" verbose="0" confidence="0" />
        <TotalSasa name="exposed_hydrophobics" confidence="0" hydrophobic=True />
        <ScoreType name="total_hydrophobic" scorefxn=TotalHydrophobic threshold=0/>
        <CalculatorFilter name=buried_np equation="total - exposed" threshold="1" confidence=0>
```

```
        <VAR name="total" filter="total_hydrophobic"/>
        <VAR name="exposed" filter="exposed_hydrophobics"/>
        </CalculatorFilter>
        <CalculatorFilter name=buried_over_exposed equation="buried / exposed" threshold="1"
confidence=0>
        <VAR name="buried" filter="buried_np"/>
        <VAR name="exposed" filter="exposed_hydrophobics"/>
        </CalculatorFilter>
        <CalculatorFilter name=bb equation="hbond / rescount" threshold="-0.23" confidence=0>
        <VAR name="hbond" filter="hbond_sfn"/>
        <VAR name="rescount" filter="nres"/>
        </CalculatorFilter>
        <CalculatorFilter name=mean_dslf equation="dslf / cyscount" threshold="-0.35" confidence=0>
        <VAR name="dslf" filter="dslf_fa13"/>
        <VAR name="cyscount" filter="nres_cys"/>
        </CalculatorFilter>
        <CavityVolume name="cavity_volume" />
        <PackStat name=pack confidence=0/>
        <Holes name="holes" confidence=0/>
        <Ddg name=ddg  scorefxn=SFXN7 threshold=-10 jump=1 repeats=1 repack=0 confidence=0/>
        <SSPrediction name="mismatch_probability" confidence="0"
cmd="/work/tlinsky/Rosetta/tools/fragment_tools/psipred/runpsipred_single" use_probability="1"
mismatch_probability="1" use_svm="0" />
    </FILTERS>
    <PROTOCOLS>
        <Add filter_name="ds_entropy" />
        <Add filter_name="res_count_core_SCN" />
        <Add filter_name="res_count_core_SASA" />
        <Add filter_name="percent_core_SCN" />
        <Add filter_name="percent_core_SASA" />
        <Add filter_name="contact_all" />
        <Add filter_name="contact_core_SCN" />
        <Add filter_name="contact_core_SASA" />
        <Add filter_name="degree" />
        <Add filter_name="entropy" />
        <Add filter_name="cavity_volume" />
        <Add filter_name="ss_sc" />
        <Add filter_name="exposed_hydrophobics" />
        <Add filter_name="holes" />
        <Add filter_name="bb" />
        <Add filter_name="mean_dslf" />
        <Add filter_name="buried_np" />
        <Add filter_name="pack" />
        <Add filter_name="mismatch_probability" />
        <Add filter_name="degree_core_SCN" />
        <Add filter_name="degree_core_SASA" />
    </PROTOCOLS>
</dock_design>
```

Supplementary Figure 5: Fold-From-Loops Scripts. (a) RosettaScripts xml file to generate *de novo* backbones around motif fragment using the specified blueprint files, and then build disulfide bonds into those backbones. (b) RosettaScripts xml file adding PD-1 to the generated *de novo* backbones, constraining the motif residues, and then doing several rounds of sequence design on the designed peptide. (c) RosettaScripts xml to optimize the designed binder sequence. (d) RosettaScripts xml to filter final designs on several monomer metrics.

(a) Step 1: Direct Segment Lookup

```
<ROSETTASCRIPTS>
	<SCOREFXNS>
		<ScoreFunction name="SFXN_STD" weights="beta_nov15" >
		</ScoreFunction>
	</SCOREFXNS>
	<MOVERS>
		<DirectSegmentLookupMover name="segment_lookup" rmsd_tolerance="0.75"
structure_store="/home/fordas/databases/vall.h5" stored_subset_name="inserted_lookup_segment"
max_insertion_length="10"/>
		<MultiplePoseMover name="minimize_segment">
		<xi:include href="minimize_segment.xml" />
		</MultiplePoseMover>
		<MultiplePoseMover name="profile_segment">
		<xi:include href="profile_segment.xml" />
		</MultiplePoseMover>
	</MOVERS>
	<APPLY_TO_POSE>
	</APPLY_TO_POSE>
	<PROTOCOLS>
		<Add mover="segment_lookup" />
		<Add mover="minimize_segment" />
		<Add mover="profile_segment" />
	</PROTOCOLS>
</ROSETTASCRIPTS>
```

minimize_segment.xml

```
<ROSETTASCRIPTS>
	<SCOREFXNS>
		<ScoreFunction name="hard_bb" >
			<Reweight scoretype="coordinate_constraint" weight="2." />
			<Reweight scoretype="cart_bonded" weight="0.5" />
		</ScoreFunction>
	</SCOREFXNS>
	<TASKOPERATIONS>
		<RestrictAbsentCanonicalAAS name="ala_only" resnum="0" keep_aas="A" />
		<OperateOnResidueSubset name="only_lookup_segment">
			<Not>
				<StoredResidueSubset
subset_name="inserted_lookup_segment" />
			</Not>
			<PreventRepackingRLT/>
		</OperateOnResidueSubset>
	</TASKOPERATIONS>
	<MOVERS>
		<PackRotamersMover name="to_ala" scorefxn="hard_bb"
task_operations="only_lookup_segment,ala_only" />
		<TaskAwareMinMover name="hardmin_bb" scorefxn="hard_bb"
type="lbfgs_armijo_nonmonotone" tolerance="0.0001" chi="1" bb="1" cartesian="1"
task_operations="only_lookup_segment" />
	</MOVERS>
	<PROTOCOLS>
		<Add mover="to_ala" />
		<Add mover="hardmin_bb" />
	</PROTOCOLS>
</ROSETTASCRIPTS>
```

profile_segment.xml

```
<ROSETTASCRIPTS>
        <RESIDUE_SELECTORS>
                <PrimarySequenceNeighborhood name="lookup_segment_span">
                        <StoredResidueSubset subset_name="inserted_lookup_segment" />
                </PrimarySequenceNeighborhood>
        </RESIDUE_SELECTORS>
        <MOVERS>
                <SegmentSequenceProfileMover name="lookup_segment_profile”
structure_store="/home/fordas/databases/vall.h5” residue_selector="lookup_segment_span”
output_pssm_inline="segment_profile" rmsd_tolerance="1.25" />
        </MOVERS>
        <PROTOCOLS>
                <Add mover="lookup_segment_profile" />
        </PROTOCOLS>
</ROSETTASCRIPTS>
```

(b) Step 2: Sequence Design & Filtering

```
<ROSETTASCRIPTS>
        <SCOREFXNS>
                <ScoreFunction name="sfxn_std" weights="beta" />
        </SCOREFXNS>
        <TASKOPERATIONS>
                <LimitAromaChi2 name="limitchi2" include_trp="1" />
                <OperateOnCertainResidues name="cst_target" >
                        <ChainIs chain="B"/>
                        <PreventRepackingRLT/>
                </OperateOnCertainResidues>
                <ReadResfile name="resfile" />
        </TASKOPERATIONS>
        <FILTERS>
                <ShapeComplementarity name="sc_filt" jump="1" verbose="1" min_sc="0.65"
write_int_area="0" confidence="1" /> #GR918 is 0.69023
                <Ddg name="ddg" scorefxn="sfxn_std" jump="1" repack="1" repeats="3" threshold="-
30" confidence="1" /> #GR918 is -30.219
                <Sasa name="sasa" threshold="1500" jump="1" confidence="1" /> #GR918 is 1498.9
        </FILTERS>
        <MOVERS>
                <FavorSequenceProfile name="pssm" weight="0.1" pssm="%%PSSM%%"
scorefxns="sfxn_std" />
                <FastDesign name="fastdes" task_operations="limitchi2,cst_target,resfile"
scorefxn="sfxn_std" clear_designable_residues="0" repeats="4" ramp_down_constraints="0" />
        </MOVERS>
        <PROTOCOLS>
                <Add mover="pssm" />
                <Add mover="fastdes" />
                <Add filter="ddg" />
                <Add filter="sc_filt" />
                <Add filter="sasa" />
        </PROTOCOLS>
</ROSETTASCRIPTS>
```

Supplementary Figure 6: Direct Segment Lookup Scripts. (a) RosettaScripts xml file running the DirectSegmentLookup mover and generating a PSSM for the grafted loops. (b) RosettaScripts xml file for sequence design and filtering of grafted loops.

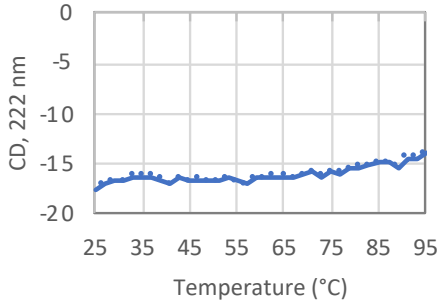
Construct	Amino Acid Sequence
GR918	CFCVCIDGPQWDYRYGNKEQCKKFLTECEQKNPGAEVYIQC
GR918.2	CFCVCITGPQWDYRYGNKEQCKKFLTECEQKNPGAEVEIQC
GR918.3	CFCVCITGPQWDFRYGNKEQCEKFLKECEQKNPGAEVEIQC
LoopGraft	CFCVCIDNSHDTWWYWGYRYGNKEQCKKFLTECEQKNPGAEVYIQC
LGm	CICVCVKTSHDARRYWKYRYGNKEQCKKFLTECEQKNPGAEVYIQC
LGm.2	CICWCVKTSPDHRRYWKYRYGNKLQCKKWLSECEQKNPGAEVNIQC
LGm2.h1	CICWCAKTKPDHRRYGKYRYGNRLQCKKWLSECAQQNPGAEVNIQC
PD-MP1	CLCWCARTKPFHRRYGKYLYGTRLQCKKWLSECAQQNPGARVNIQC
Spytag_PD-MP1	GSAHIVMVDAYKPTKGDGGKGSDGEQKLISEEDLGKGSGGSSGCLC WCARTKPFHRRYGKYLYGTRLQCKKWLSECAQQNPGARVNIQC
Spycatcher_2L6HC3_13	GAMVDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGAT MELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAIT FTVNEQGQVTVNGKATKGDAHILEGLNDIFEAQKIEWHEGSGSGTKY ELRRALEELEKALRELKKSLDELERSLEELEKNPSEDALVENNRLNVENN KIIVEVLRIIAEVLKIIAKSD
PD-MP1_2L6HC3_13	**GSAHIVMVDAYKPTKGDGGKGSDGEQKLISEEDLGKGSGGSSGCI CWCTKTVPDGRRYWKYRYGNKLICKKWLSECQQKNPGAEINIQC MGSSHHHHHHSSGLVPRGSHMS**GAMVDTLSGLSSEQGQSGDM TIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDF YLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHIL EGLNDIFEAQKIEWHEGSGSGTKYELRRALEELEKALRELKKSLDELERS LEELEKNPSEDALVENNRLNVENNKIIVEVLRIIAEVLKIIAKSD

Supplementary Figure 7: Amino acid sequences for all described constructs. **=Spytag/Spycatcher conjugation.

(a) Data Collection & Refinement Statistics

Wavelength	0.9791
Resolution range	25.4 - 1.07 (1.108 - 1.07)
Space group	C 1 2 1
Unit cell	51.849 26.744 51.537 90 99.668 90
Total reflections	127753 (11506)
Unique reflections	29635 (2856)
Multiplicity	4.3 (4.0)
Completeness (%)	97.40 (94.72)
Mean I/sigma(I)	12.03 (2.40)
Wilson B-factor	10.97
R-merge	0.05734 (0.4592)
R-meas	0.06513 (0.5299)
R-pim	0.03024 (0.258)
CC1/2	0.998 (0.887)
CC*	0.999 (0.97)
Reflections used in refinement	29635 (2852)
Reflections used for R-free	1455 (148)
R-work	0.1455 (0.1990)
R-free	0.1498 (0.2117)
CC(work)	0.971 (0.945)
CC(free)	0.980 (0.899)
Number of non-hydrogen atoms	826
macromolecules	738
solvent	88
Protein residues	85
RMS(bonds)	0.009
RMS(angles)	1.38
Ramachandran favored (%)	96.30
Ramachandran allowed (%)	3.70
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.00
Clashscore	4.99
Average B-factor	15.07
macromolecules	13.80
solvent	25.77

(b) GR918.2 Thermal Melt



Supplementary Figure 8: Crystal diffraction statistics and thermal stability. (a) Data collection and refinement statistics for crystal structure. (b) Thermal denaturation of GR918.2, monitoring CD signal at 222 nm.