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2 **Destabilisation of the human RED-SMU1 splicing complex**
3 **as a basis for host-directed anti-influenza therapy**
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33
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35 Major : Biological Sciences

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1 **Abstract**

2

3 New therapeutic strategies targeting influenza are actively sought due to limitations in current
4 drugs available. Host-directed therapy is an emerging concept to target host functions
5 involved in pathogen life cycles and/or pathogenesis, rather than pathogen components
6 themselves. From this perspective, we focused on an essential host partner of influenza
7 viruses, the RED-SMU1 splicing complex. Here we identified two synthetic molecules
8 targeting an α -helix-groove interface essential for RED-SMU1 assembly. We solved the
9 structure of SMU1 N-terminal domain in complex with RED or bound to one of the molecules
10 identified to disrupt this complex. We show that these compounds inhibiting RED-SMU1
11 interaction also decrease endogenous RED-SMU1 levels, inhibit viral mRNA splicing and
12 viral multiplication, whilst preserving cell viability. Overall, our data demonstrate the potential
13 of RED-SMU1 destabilizing molecules as a novel antiviral therapy, which could be active
14 against a wide range of influenza viruses and be less prone to drug resistance.

15

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17 **Keywords** : Influenza virus, Splicing, Structure-based drug screening, Host-directed
18 antivirals

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1 **Significance Statement**

2

3 Influenza virus is a serious threat to global public health and there is a critical need for
4 innovative anti-influenza drugs. Two broad, non-exclusive approaches to inhibit viral
5 replication are possible, either targeting directly viral proteins or targeting host proteins
6 essential for the viral life cycle. Here we took the second approach, which is more likely to
7 counter the problem of drug-resistant virus emergence. Focusing on an essential host
8 partner of influenza viruses, the RED-SMU1 splicing complex, we performed a virtual
9 structure-based drug screening. We identified two synthetic molecules which interfere with
10 RED-SMU1 complex assembly, inhibit the splicing of viral messenger RNAs, and show
11 potential for the inhibition of influenza virus infections.

12 **\body**

1 **Introduction**

2 The increasing incidence of drug-resistant pathogens calls for the development of novel
3 therapeutic strategies. In recent years, the concept of host-directed therapies, which target
4 host determinants essential for the infectious life cycle and/or pathogenesis rather than
5 pathogens components, has been rapidly expanding (1, 2). Preclinical studies suggest they
6 could show clinical safety while providing the advantage of broad spectrum efficacy and
7 reduced antiviral resistance. A range of host-directed therapies for several bacterial and viral
8 diseases, with different mechanisms of action, are currently in clinical trials (1, 2).

9 Influenza A viruses (IAVs) are a leading cause of morbidity and mortality worldwide, as they
10 are responsible for recurring annual epidemics, frequent epizootics, and occasional
11 pandemics (3). The drugs currently licensed for treatment of influenza target viral
12 components (the M2 ion channel, the neuraminidase and the polymerase) and they all lead
13 to the emergence of resistance variants. The M2 inhibitors are no longer recommended for
14 use since currently circulating human H3N2 and H1N1pdm09 viruses have become naturally
15 resistant to these drugs (4). Although the frequency of resistance to neuraminidase inhibitors
16 (NAIs) of currently circulating human IAVs remains low (around 0.5%) (5), large clusters of
17 H1N1pmd09 viruses resistant to oseltamivir, the most widely used NAI, have been observed
18 (6, 7). The former seasonal H1N1 IAVs had become globally oseltamivir-resistant during the
19 2007-2008 season (8). Favipiravir, a purine nucleoside analog also known as T-705, is
20 undergoing phase III trials in America and Europe and has been approved in Japan to treat
21 pandemic influenza virus infections. In October 2018, FDA approved Xofluza, a selective
22 inhibitor of the polymerase PA subunit, for the treatment of acute uncomplicated influenza.
23 However, the first evidence for viral adaptation to favipiravir treatment in cell culture has
24 been reported recently (9, 10), and the emergence of PA variants with a mutation conferring
25 resistance to Xofluza was observed in 9.7% of treated patients in the Phase III trial (11). In
26 this context, novel anti-influenza drugs are actively being sought to efficiently fight IAVs,
27 particularly with regard to pandemic preparedness (12, 13). Antivirals currently in late-phase
28 clinical trials include monoclonal antibodies against the viral hemagglutinin or matrix protein

1 (14, 15), a selective inhibitor of the polymerase PB2 subunit (16), and two compounds
2 targeting host cell factors: nitaxozanide, which impairs trafficking and maturation of the viral
3 hemagglutinin (HA) (17, 18); and DAS181, which enzymatically removes the membrane
4 receptors required for IAV attachment to target cells (19, 20). Continuous progress on the
5 identification of host factors involved in IAV life cycle provides a basis for the development of
6 alternative host-directed antiviral drugs (21, 22). Following this approach we have
7 characterized the structure of a human splicing factor, RED-SMU1, that is essential for IAV
8 life cycle, and exploited the structural data to investigate this cellular factor as a potential
9 target for IAV therapy.

10 Infectious IAV particles contain eight ribonucleoprotein complexes (vRNPs), corresponding to
11 a set of eight distinct viral genomic RNA (vRNA) segments encapsidated with nucleoproteins
12 (NP) and associated with the heterotrimeric viral RNA-dependent RNA polymerase (FluPol)
13 consisting of the PB1, PB2 and PA subunits (3). The viral genome has a limited size (about
14 13.5 kb), but IAVs have evolved a variety of strategies to expand their coding capacity. Viral
15 mRNAs are synthesized by the FluPol in the nucleus of infected cells. Although most of
16 these are intronless, the M, NS and PB2 segments produce both unspliced mRNAs (M1,
17 NS1, PB2) and spliced mRNAs (M2, NS2, PB2-S1). We previously showed that FluPol
18 recruits a complex formed by the human splicing factors RED (78.9 kDa) and SMU1 (57.5
19 kDa) by direct binding to RED (23). RED-SMU1 regulates the splicing of viral NS1 mRNA
20 into the mRNA encoding the multifunctional and essential NS2/NEP protein. In cells depleted
21 for RED or SMU1, the production of infectious influenza virions showed a 100-fold reduction
22 (23), designating the RED-SMU1 complex as a promising IAV drug target.

23 The RED and SMU1 factors are associated to the pre-catalytic spliceosome (24). They were
24 found to jointly regulate the splicing of specific cellular pre-mRNAs, with a possible role in the
25 selection of cryptic 5' splice sites (25-27). As we and others have observed that RED and
26 SMU1 directly bind (23, 26, 28) and stabilize each other (23, 26, 27), a significant proportion
27 of functional RED-SMU1 complexes is likely to exist.

1 Here we performed subdomain analysis of human RED and SMU1 proteins using cell-based
2 interaction assays, and solved the crystal structure of a minimal RED-SMU1 complex at 3.0
3 Å resolution. The structure shows similarities to the recently published RED and SMU1
4 orthologs in *Caenorhabditis elegans* (ceRED and ceSMU1) (29) but reveals a more intricate
5 RED-SMU1 interface. Based on our structural findings, we identified small molecules that
6 target an α -helix-groove interface essential for RED-SMU1 interaction. Lastly, we provide
7 supporting data to demonstrate the potential of such RED-SMU1 destabilizing molecules for
8 the inhibition of IAV infections.

9

1 Results

2

3 *Delineation of a minimal RED-SMU1 complex*

4 We previously found that the N-terminal domain of RED (residues 1 to 315) binds to SMU1
5 as efficiently as the full-length RED protein (23). Using a split-luciferase based interaction
6 assay, we further delineated a minimal human RED-SMU1 complex. Plasmids encoding
7 various subdomains of RED tagged with the Gluc2 fragment of *Gaussia princeps* luciferase
8 were co-transfected with plasmids for the expression of SMU1 or SMU1_{Nter} (residues 1-196)
9 fused to the trans-complementing *Gaussia princeps* luciferase Gluc1 fragment. The rationale
10 for RED truncations shown in **Fig. 1A** was based on secondary structure and disorder
11 predictions (**SI Appendix, Fig. S1**), with an iterative testing and design approach. As
12 revealed by the relative luciferase activities measured in cell extracts, the RED[206-260]
13 subdomain (named thereafter RED_{mid}) retained the interaction with SMU1 (black bars) or
14 SMU1_{Nter} (grey bars) (**Fig. 1A**). The SMU1 interaction signals were almost 8-fold higher with
15 RED_{mid} than with the full-length RED ($p < 0.01$), and were higher than with any longer
16 truncated version of RED. Western blot analysis of the cell lysates used for luciferase assay
17 showed that this higher interaction signal was not due to a higher level of expression of
18 RED_{mid} (**Fig. 1B**, black arrowhead) compared to other deletion mutants of RED or to the full-
19 length RED (open arrowhead). A likely explanation is that the short RED_{mid} subdomain is
20 more stable thermodynamically than the longer versions of RED and that it becomes more
21 accessible upon removal of the adjacent polypeptides. For several other protein pairs, it was
22 indeed previously shown that interactions are more easily detected when using isolated
23 interacting subdomains or peptides (30). Notably, the RED[219-299] subdomain produced
24 lower interaction signals compared to RED_{mid} but also to the full-length RED protein,
25 suggesting that residues 206 to 218 of RED are essential for the interaction with SMU1.

26

27

1 **The 3D structure of human RED_{mid}-SMU1_{Nter} reveals two molecular interfaces**

2 Based on our cell-based interaction results, we set up a co-expression strategy to produce
3 and pull-down the human RED_{mid}-SMU1_{Nter} complex, using a hexa-histidine tag fused to the
4 N-terminus of RED_{mid}. The recombinant complex was crystallized and the structure solved by
5 molecular replacement using the X-ray structure of the SMU1_{Nter} alone without RED_{mid}
6 obtained earlier (**SI Appendix, Fig. S2 and Table S1**). The final model was built using the
7 anomalous signal of a Se-met derivative dataset for an accurate sequence assignment. The
8 asymmetric unit is composed of sixteen molecules: eight SMU1_{Nter} molecules that assemble
9 into four dimers, and eight RED_{mid} molecules each one being associated with a SMU1_{Nter}
10 monomer. The X-ray structure reveals two distinct interfaces between SMU1_{Nter} and RED_{mid}
11 (**Fig. 2A**). First, the region of RED_{mid} corresponding to residues 235-257 disrupts the initial
12 fold of the N-terminus of SMU1_{Nter} to form a three-stranded antiparallel β -sheet, that is
13 uniquely stabilized by main chain interactions (Interface I, **Fig. 2B**). Second, the helical
14 region of RED_{mid} (corresponding to residues 211-221) lies in a hydrophobic groove delimited
15 by three continuous α -helices (α 4 to α 6) on the surface of SMU1_{Nter} (Interface II, **Fig. 2C**).
16 The structural results are consistent with the high and low interaction signals measured with
17 the SMU1-RED[206-260] and SMU1-RED[219-299] combinations, respectively (**Fig. 1A**).
18 The comparison of our human RED_{mid}-SMU1_{Nter} structure with the recently published
19 structure of a *C. elegans* minimal RED-SMU1 complex ((29) and **SI Appendix, Fig. S3**) is
20 discussed below. Importantly, the human structure suggests that SMU1 needs to form a
21 dimer to assemble with RED, as Interface I extends across two distinct SMU1_{Nter} monomers.
22 In agreement with this observation, SEC-MALLS analysis on purified full-length SMU1
23 protein showed that it is mostly dimeric (**Fig. S4A**), and treatment of A549 or HEK-293T cells
24 with the cell-permeable crosslinking agent disuccinimidyl suberate (DSS) followed by
25 western blotting revealed that the endogenous SMU1 protein forms dimers in live cells (**Fig.**
26 **S4B**).

27

28

1 ***Mutational analysis provides a rationale for targeting the α -helix:groove interface***

2 To confirm our structural data and assess the druggability of the RED-SMU1 interface, we
3 performed structure-based mutational analysis of the full-length RED and SMU1 proteins.
4 Point mutations were introduced at interface I (**Fig. 2B**) or II (**Fig. 2C** and **Fig. 3A-B**). Split-
5 luciferase complementation assays were performed and expression levels of the mutants
6 were assessed (**Fig. 3C-H**). Short deletions in the domains of SMU1 (Δ 3-7) or RED (Δ 237-
7 241 or Δ 254-256) involved in interface I reduced the RED-SMU1 interaction signal up to 3-
8 fold (**Fig. 3C**, black bars). In the presence of SMU1 mutations targeting the interface II
9 (D57A-E89A, L60D-I63D, L73D-Y77D, L84D-L87D) or the hinge between interface I and II
10 (P157T-P158T), a 2- to 10-fold reduction of the interaction signal was also observed (**Fig.**
11 **3D**). Notably, no cumulative effect was observed when deletions at interface I were
12 combined with the SMU1 mutations D57A-E89A destabilizing interface II (**Fig. 3C**, grey
13 bars), suggesting that both molecular interfaces are required to stabilize the RED-SMU1
14 complex. RED mutants L212D, V216D and L220D targeting interface II showed a 2-fold
15 reduction of the interaction signals with SMU1 or SMU1-D57Q (**Fig. 3E**). In contrast the
16 RED-N215D and M219D mutations had little effect on the interaction with SMU1 (**Fig. 3E**),
17 as expected from the 3D structure (**Fig. 3A-B**). Together, these results confirm our structural
18 findings, demonstrate that a mere disruption of interface II α -helix:groove impairs the stability
19 of the RED-SMU1 complex, and point to SMU1 D57, L60, I63, L73, Y77, L84, L87 and E89
20 as key RED binding residues.

21

22 ***Disrupting the RED-SMU1 complex: proof-of-principle by overexpressing RED_{mid}***

23 Based on the data presented above, we reasoned that if overexpressed, the RED_{mid} helical
24 domain should efficiently compete with the full-length RED protein for binding to the surface
25 groove on SMU1, and thereby disrupt the RED-SMU1 complex. As a control, we used a
26 RED_{mid}-V216D mutant, which accumulates at the same levels as RED_{mid} but is strongly
27 impaired for SMU1 binding. Of note, the effect of V216D mutation on SMU1 binding is much
28 stronger in the context of RED_{mid} (> 99% reduction) compared to the full-length RED (about

1 50% reduction) (**SI Appendix, Fig. S5A**). Upon overexpression of the mCherry-RED_{mid}
2 fusion protein we observed a >2-fold reduction of the RED-SMU1 interaction signal, as
3 measured with the split-luciferase complementation assay, while with the control mCherry-
4 RED_{mid}-V216D mutant, no such reduction was observed ($p < 0.01$, **SI Appendix, Fig. S5B-C**).
5 We then assessed the effect of RED_{mid} overexpression on the endogenous RED-SMU1
6 complex. As there is evidence that RED and SMU1 stabilize each other (23, 26, 27), RED-
7 SMU1 disruption is expected to result in decreased steady-state levels of the proteins. We
8 observed a decrease in endogenous cellular levels of RED when overexpressing RED_{mid},
9 whereas empty vectors or RED_{mid}-V216D had no effect (**Fig. S5D**, upper panel). However,
10 SMU1 levels remained unchanged (**Fig. S5D**, middle panel). A likely interpretation is that the
11 SMU1 protein complexed with RED_{mid} remains stable, whereas the dissociated RED protein
12 undergoes degradation.

13 Next we monitored the effect of RED_{mid} overexpression on the replication of a recombinant
14 A/WSN/33 virus carrying a luciferase reporter gene (WSN-PB2-Nanoluc). The luciferase
15 activity measured in cell lysates prepared at 24 hours post-infection (hpi), was lower in cells
16 overexpressing RED_{mid} compared to cells transfected with the empty pCI vector or
17 overexpressing RED_{mid}-V216D (**Fig. S5E**). On the contrary, there was no significant
18 difference between cells transfected with the empty pCI vector and those overexpressing
19 RED_{mid}-V216D. Furthermore, splicing of the viral NS1 mRNA into NS2 mRNA, which is
20 dependent upon RED-SMU1 (23), was reduced in RED_{mid}-expressing cells compared to the
21 RED_{mid}-V216D control (**Fig. S5F**). Overall, our findings strengthen the rationale of targeting
22 the RED-SMU1 α -helix:groove interface to inhibit IAV replication.

23

24 ***In silico identification and evaluation of RED-SMU1 disrupting compounds***

25 We used the available structural information on the RED_{mid}-SMU1_{Nter} complex to perform
26 molecular docking-based screening for compounds targeting the RED-binding groove at the
27 surface of SMU1_{Nter}. The virtual high-throughput screening (vHTS) was performed on a set of
28 4,121 chemical compounds from our in-house chemical database (LCPBT-DB), comparable

1 to the Prestwick or FDA-approved libraries in terms of chemical diversity (see **SI Appendix**).

2 A flow chart for the vHTS and compound selection pipeline is represented in **Fig. 4A**. To

3 ensure a time/precision ratio compatible with vHTS we used a protocol in which amino acid

4 side chains of the protein are left flexible only around the binding site, and we tested 10

5 random conformers for each ligands. The resulting poses were ranked according to their

6 docking scores, as described in the Methods section. The poses with the highest docking

7 scores were further filtered to select for compounds that are predicted to bind at least 4 of the

8 8 SMU1 residues required for efficient binding to RED: D57, L60, I63, L73, Y77, L84, L87,

9 E89 (**Fig. 4B** and **Fig. 3D**). A total of 37 compounds fulfilled both criteria, and a subset of 16

10 molecules with representative chemical scaffolds were further evaluated.

11 Among these, 14 showed no or only limited cytotoxicity at 60 μ M, and were further tested at

12 the same concentration in cell-based assays for the inhibition of RED-SMU1 interaction and

13 IAV replication. Compounds inhibiting RED-SMU1 interaction by more than 20%, and IAV

14 replication by more than 80% were selected for further evaluation. In total, three compounds

15 showed promising inhibitory effect in both assays (**Fig. 4A**). Of these, one molecule, LSP641

16 (**Fig. 4C**), was successfully co-crystallised with the purified recombinant SMU1_{Nter} domain.

17 Structural data showed that LSP641 locates in the hydrophobic RED binding pocket of SMU1

18 as expected (**Fig. 4D**) and is engaged in several molecular interactions with SMU1 (**Fig. 4E**).

19 The 2-amino-pyrimidine group present in LSP641 forms key hydrogen bonds with Q61 and

20 Q64, whereas the urea group forms a hydrogen bond with Y77. In the apolar region of

21 SMU1, D57, L60 and L96 are involved in Pi-Pi or σ -Pi interactions, in particular with the

22 pyridopyrimidine scaffold of LSP641. Furthermore, W56, I63, L84 and A92 form hydrophobic

23 interactions with LSP641. Remarkably, the LSP641 molecule interacts with D57, L60, I63,

24 Y77 and L84, *i.e.* 5 of the 8 residues shown earlier to be involved in RED-SMU1 interaction,

25 to account for the inhibition of this protein-protein interaction.

26 Guided by the SMU1_{Nter}:LSP641 co-structure, we selected 27 LSP641-related compounds

27 and we filtered them through a second evaluation round (**Fig. 4A**), which led to the selection

28 of LSP61 (31) (**Fig. 4C and 4F**). Unfortunately, we were unable to co-crystallize LSP61 with

1 SMU1_{Nter}. This different behavior of LSP61 compared to LSP641 might be related to the
2 absence/presence of stacking interactions in the co-crystal. However, *in silico* molecular
3 docking supported binding interactions similar to LSP641 (**Fig. 4G**). Hydrogen bonds with
4 Q64 and Y77 are conserved, as well as Pi- or σ -Pi-interactions with D57 and L60, and
5 hydrophobic interactions with W56 and L84. Q61 is involved in Pi-interactions, whereas V41,
6 V80 and L96 are forming novel hydrophobic interactions. Most importantly, the
7 benzylpiperidine group of LSP641 that is not binding SMU1 in the crystal structure (**Fig. 4D-**
8 **E**), is replaced in LSP61 by an alkyl chain which interacts with A62 and V41 in a neighboring
9 hydrophobic pocket of SMU1 and therefore optimizes molecular recognition (**Fig. 4F-G**). This
10 led us to select LSP61 to conduct further investigations, in parallel with LSP641.

11 LSP61 and LSP641 were compared in the RED-SMU1 interaction assay across the 0.23 to
12 60 μ M concentration range. The EC50 (half-maximal effective concentration) was estimated
13 to be 15 μ M for LSP61 ; LSP641 showed a milder effect resulting in a 40% inhibition at 60
14 μ M, compared to 90% with LSP61, and a plateauing curve (**Fig. 5A**). When a similar
15 FOS:JUN interaction assay was used as a specificity control, none of the two compounds
16 showed any significant effect (**Fig. 5B**). The Kd of the SMU1_{Nter}-LSP61 complex could not be
17 determined, most likely due to the low solubility of LSP61 (logP=4.8). The effect of LSP61 on
18 the endogenous RED-SMU1 complex was assessed using the steady-state levels of RED
19 and SMU1 as a proxy. Expression levels of both proteins decreased when treating cells with
20 15, 30 and 60 μ M LSP61 (**Fig. 5C**). At these concentrations, no cytotoxicity but some
21 cytostatic effect was observed as assessed by the steady levels of ATP measured in culture
22 wells (a proxy for cell count) upon 24 and 48 hours of incubation with LSP61 (**Fig. 5D**) and
23 the observation of cell monolayers by bright field microscopy (**SI Appendix, Fig. S6**).

24

25 ***LSP61 inhibits IAV replication***

26 Finally we assessed the ability of LSP61 to inhibit IAV replication. We first used the WSN-
27 PB2-Nanoluc virus, in parallel with a recombinant human Respiratory Syncytial Virus (RSV)
28 expressing Firefly luciferase as a specificity control (RSV replicates in the cytoplasm with no

1 direct involvement of the nuclear splicing machinery postulated). After viral adsorption to
2 A549 cells, the LSP61 or LSP641 compounds were added to the cells across the 7.5 - 60 μ M
3 concentration range, and the luciferase activities were determined at 24 hpi. The RSV-Firefly
4 signals showed only moderate reductions (**Fig. 6A**, grey bars). In contrast the WSN-Nanoluc
5 signals showed a significant, dose-dependent reduction, more pronounced for LSP61 (25-
6 fold at 60 μ M, $p < 0.0001$) compared to LSP641 (6-fold at 60 μ M, $p < 0.005$) (**Fig. 6A**, black
7 bars). Growth kinetics were performed with wild-type IAVs (the WSN virus and
8 representatives of H1N1pdm09 and H3N2 circulating human IAVs) in the presence of 60 μ M
9 LSP61 and the supernatants were titrated by plaque assay. Because the H1N1pdm09 and
10 H3N2 isolates grew poorly on A549 cells, canine MDCK-SIAT cells (32) were used in these
11 experiments. In the presence of LSP61 the production of infectious particles showed a 10- to
12 100-fold reduction at 24 and 48 hpi (**Fig. 6B**). In comparison, on A549 cells, the production of
13 WSN infectious particles was reduced about 1000-, 100- and 10-fold at 24, 48 and 72 hpi,
14 respectively (**Fig. 6C**). In MDCK-SIAT cells, canine Mx proteins lack anti-influenza activity,
15 which limits the establishment of a strong interferon-induced antiviral state and favors a very
16 efficient replication of influenza viruses (33). This particular feature of MDCK-SIAT cells likely
17 accounts for the fact that a strong inhibition with the LSP61 compound was more difficult to
18 achieve compared to in A549 cells.

19 In WSN-infected cells, the LSP641 and LSP61 compounds inhibited the splicing of the viral
20 NS1 mRNA into NS2 mRNA at 60 μ M concentration. Indeed, the NS2 to NS1 mRNA ratio
21 was reduced by 30% with LSP641, and 50% with LSP61 (**Fig. 6D**), which is comparable to
22 previous results obtained when either RED or SMU1 was knocked-down in infected cells
23 (23). In contrast LSP641 and LSP61 did not inhibit the splicing of the viral M1 mRNAs (**Fig.**
24 **S7A**), in agreement with our previous findings that M1 mRNA splicing is little affected by
25 RED or SMU1 silencing compared to NS1 mRNA splicing (23). Likewise, PB2 mRNA
26 splicing, whose role in viral infection remains unclear (34), is not affected by 60 μ M LSP641
27 or LSP61 (**Fig. S7B**). The specificity of LSP641 and LSP61 towards the splicing of NS1
28 mRNA, taken together with our findings that both compounds inhibit RED-SMU1 interaction

1 (Fig. 5A) and IAV replication (Fig. 6A), with the observed effects being consistently more
2 pronounced for LSP61, strongly argue for their antiviral effect being due to disruption of the
3 RED-SMU1 complex.

4

5

1 Discussion

2 We have determined the crystal structure of the interacting human SMU1 N-terminal region
3 (SMU1_{Nter}) and RED central region (RED_{mid}). As expected from the high conservation of
4 these two protein subdomains, the observed structure shows similarity to the one reported
5 for *C. elegans* (29). In both structures SMU1_{Nter} assembles into a dimer through inter-
6 molecular contacts between LisH motifs (**SI Appendix, Fig. S2**). Interestingly our structure
7 reveals a more intricate SMU1-RED interaction with two binding interfaces, including a β -
8 sheet: β -sheet interface (not present in the *C. elegans* structure) in addition to an α -
9 helix:groove (**SI Appendix, Fig. S3**). Unlike Ulrich *et al.* who assembled complexes from
10 purified RED and SMU1 subdomains produced separately in *E. coli* (29), we chose a co-
11 expression strategy which allowed us to first delineate in HEK-293T cells and then to co-
12 purify from *E. coli* a very stable RED_{mid}-SMU1_{Nter} complex. RED and SMU1 are part of the
13 spliceosomal pre-catalytic B complex, whose molecular architecture was very recently
14 elucidated by cryo-EM (35). Fitting of our atomic structure of RED_{mid}-SMU1_{Nter} enabled us to
15 refine the cryo-EM 3D model by applying the conformational constraint imposed by the newly
16 revealed β -sheet: β -sheet interface between RED_{mid} and SMU1_{Nter}. The model (**SI Appendix,**
17 **Fig. S8**) highlights a major structural role for RED, as it is positioned at the interface of the
18 SMU1 LisH motif-based dimerization domain and the SF3B1 core spliceosomal factor. This
19 pivotal position could contribute to a stabilization of the B complex structure during the early
20 stages of spliceosome assembly that precede catalytic activation.

21 Based on our human RED_{mid}-SMU1_{Nter} structure, we designed pilot experiments and *in silico*
22 screening which led to the identification of two inhibitors, LSP641 and LSP61; they disrupt
23 the RED-SMU1 interaction, reduce RED-SMU1 endogenous levels, inhibit IAV NS1 mRNA
24 splicing and decrease the production of infectious IAV particles. These findings add to a
25 growing list of more than 40 protein-protein interactions that have been successfully targeted
26 with small drug-like molecules, at least *in vitro* (36, 37). Several inhibitors have entered
27 clinical trials, including inhibitors of the p53-MDM2 interaction (38) and inhibitors of the
28 interaction between the HIV-1 integrase and its cofactor LEDGF (39). Prospects for

1 developing inhibitors seem to be higher for protein-protein interactions in which residues
2 critical for the interaction are concentrated in small binding pockets, as is the case for the α -
3 helix:groove interface between RED_{mid} and SMU1_{Nter}. In the recent years much progress has
4 also been made to enhance the therapeutic potential of short cell-permeable peptides as an
5 alternative to conventional therapeutic small molecules (40). In principle a peptide
6 corresponding to the short RED[211-222] α -helix could be considered as a potential inhibitor
7 of RED-SMU1 interaction. However the strong hydrophobicity of this α -helix is likely to
8 prevent its solubilisation and to preclude such a strategy from being successful.

9 The potential toxicity associated with host-directed therapy also needs to be carefully
10 evaluated. Cultured A549 cells treated with RED-SMU1 targeting compounds LSP461 and
11 LSP61 (this study), or treated with RED-SMU1 targeting siRNAs with a depletion efficiency of
12 90% (23) did not show cell death. Consistently, RED or SMU1 knock-outs in *C. elegans* or *A.*
13 *thaliana* are viable (25, 27). However, a cytostatic effect of compound LSP61 was observed,
14 as evidenced by bright field imaging showing a lower density of the cell layer in the absence
15 of dead cells, and ATP quantification supporting a slower accumulation of metabolically
16 active cells in culture wells. This observation is in agreement with previous reports showing
17 that RED and SMU1 jointly regulate alternative splicing of a subset of pre-mRNAs involved in
18 development, apoptosis and cell survival (25-27, 41, 42). The transcriptomic profiling of cells
19 treated with compound LSP61 (or depleted for RED-SMU1 as a reference) will provide a
20 means to investigate how LSP61 affects the expression and splicing of cellular genes, and
21 detect potential adverse effects to guide further drug development (43). Beyond their splicing
22 function, RED and SMU1 are associated with the mitotic spindle (44) and chromatin (45),
23 respectively, and are involved in the control of cell division (44, 46). The dual function of
24 RED-SMU1 raises the question whether our observed anti-viral effect of compounds LSP461
25 and LSP61 could be related not only to inhibition of viral mRNA splicing (as indicated by a
26 reduced NS2 to NS1 mRNA ratio upon treatment) but also cell cycle arrest. Although this
27 possibility cannot be formally excluded, it seems unlikely as IAV infection *per se* has been
28 shown to induce G0/G1 cell cycle arrest through inhibition of the RhoA/pRb signaling

1 pathway (47) and down-regulation of cyclin D3 levels (48) in cultured cells. Besides, the
2 tissue naturally targeted by IAVs, *i.e.* the airway epithelium, is essentially quiescent in
3 healthy hosts (49). However, mitogenic stimulation through intra-tracheal administration of
4 the keratinocyte growth factor in mice was found to induce the proliferation of alveolar type II
5 cells (normally quiescent by more than 99%), and this increased their susceptibility to IAV
6 infection (50). The authors suggest that the enhanced mortality due to influenza in infants or
7 cigarette smokers could be related to a higher fraction of proliferating alveolar type II cells in
8 these patients. Under this hypothesis, compounds targeting RED-SMU1 might show a
9 double effect of inhibiting IAV replication through splicing modulation, and limiting the
10 intrapulmonary spread of IAV infection through cytostatic activity.

11 Over the last two decades a growing number of small molecules that inhibit the spliceosome
12 assembly or function have been identified, but many of them have yet to be characterized
13 regarding their target and mechanism of action. The most documented are SF3B1 inhibitors
14 of the spliceostatins, pladienolides and sudemycins families (51-53). Although these drugs
15 target a core splicing factor, there is evidence for a certain degree of selectivity. SF3B1
16 inhibitors affect splicing of pre-mRNAs associated with cell cycle regulation and apoptosis,
17 and inhibit growth of cultured cancer cell lines at nM concentrations while cells derived from
18 normal tissues are less sensitive (for a review see (54, 55)). Preclinical studies have
19 suggested that splicing modulation by SF3B1 inhibitors can be well tolerated *in vivo* and
20 show potential for the treatment of several types of cancers (56-58). Taking these data into
21 account, together with the absence of observed cytotoxicity of LSP641 and LSP61 in our
22 experiments, and with the fact that a topical and short time course treatment can be
23 considered in the case of acute IAV infections, compounds targeting the RED-SMU1
24 complex can reasonably be expected to show clinical safety and efficacy in influenza
25 therapy. Our SMU1_{Nter}-LSP641 co-structure provides a valuable basis for chemical
26 optimization in order to select compounds with improved activity, specificity and
27 pharmacological properties, which can be tested *in vivo* in preclinical animal models for IAV
28 infection. Such optimized RED-SMU1 disrupting compounds could be active against a wide

1 range of IAVs, and be less likely to select for resistance mutants. Interestingly, RED and
2 SMU1 were identified as hits in two high-throughput screens looking for cellular factors
3 involved in HIV-1 life cycle (59, 60). SMU1 was also found to be associated to the E6 protein
4 of the high-risk HPV18 human papillomavirus (61). Therefore RED-SMU1 disrupting
5 compounds might not only be active against IAVs but against a wider range of viruses that
6 make use of the RED-SMU1 splicing complex. Besides potential applications as antivirals,
7 our small molecule inhibitors might also serve as tools to dissect the early stages of
8 spliceosome assembly, and lead to potential applications as anti-cancer agents (54, 62, 63).
9

1 **Materials and Methods**

2

3 **Cells, viruses, plasmids and reagents.** See **SI Appendix** for detailed information

4

5 ***In cellulo split luciferase-based interaction assays***

6 The split-luciferase protein complementation assays were performed as described in (64).
7 Briefly, HEK-293T or A549 cells were seeded in 96-well white opaque plates (Greiner Bio-
8 One) and co-transfected with 100 ng of each indicated pGluc1-P1 and pGluc2-P2 plasmids,
9 where P1 and P2 represent proteins or protein subdomains of interest. The polyethylenimine
10 PEI (Polysciences Inc) and JetPRIME (Polyplus Transfection) transfection reagents were
11 used with HEK-293T and A549 cells, respectively. Normalized luciferase values were
12 determined as described in (23, 64). At 24 h post-transfection, the luciferase enzymatic
13 activities were measured using the *Renilla* luciferase assay system (Promega) and a
14 Berthold Centro XS luminometer. When cell-based interaction assays were performed for
15 compound screening, control HEK-293T cells transfected with a full-length Gaussia
16 luciferase expression plasmid were incubated in parallel with tested compounds or DMSO
17 alone. The split-luciferase values were normalized with respect to control full-length
18 luciferase signals.

19

20 ***Protein expression in E. coli and protein purification***

21 The pETM11-SMU1_{Nter} plasmid was transformed in *E. coli* BL21-CodonPlus (Agilent).
22 Cultures were grown at 37°C in LB containing kanamycin (30 µg/mL) and chloramphenicol
23 (34 µg/mL). When the OD_{600nm} reached 0.6-0.8, the cultures were cooled down to 18°C and
24 expression induced with 0.3 mM isopropyl-thiogalactopyranose (IPTG). The cultures were
25 incubated overnight at 18°C before centrifugation. For the SMU1_{Nter}-RED_{mid} complex, a co-
26 expression strategy was set up. The pETM11-RED_{mid} plasmid was transformed in *E. coli*
27 BL21-RIL-SMU1_{Nter} competent cells and expression performed using the protocol described
28 above. Cell pellets were resuspended in lysis buffer (50 mM Hepes pH7.5, 150-500 mM

1 NaCl, 1 mM β -mercaptoethanol (β me)), and sonicated. After centrifugation, the supernatant
2 was complemented with imidazole to reach 25mM and loaded on a nickel affinity column
3 (NiNTA, GIAGEN). The resin was washed with a high salt buffer (50 mM Hepes pH7.5, 1 M
4 NaCl, 1 mM β me). The recombinant proteins were eluted with elution buffer (50 mM Hepes
5 pH7.5, 1 M NaCl, 1 mM β me, 300mM imidazole). Protein were dialyzed with TEV protease
6 overnight against the buffer without imidazole, loaded on a second NiNTA column,
7 concentrated and loaded on a size exclusion column, a HiLoad 16/600 Superdex 75 (GE
8 Healthcare) for SMU1_{Nter} or a Superdex 200 Increase 10/300GL (GE Healthcare) for the
9 SMU1_{Nter}-RED_{mid} complex. Fractions of interest were concentrated between 5 and 15 mg/mL.
10

11 **Structure determination**

12 For SMU1_{Nter}, the crystals (native and Se-Met derivative) were obtained in 0.1 M Bis-Tris
13 pH5.5, 16-20 % PEG 10K, 0.1 M ammonium acetate and cryoprotected in the same solution
14 + 30 % glycerol. Data were processed with the XDS package (65). For experimental phasing
15 of SMU1_{Nter} a highly redundant single wavelength anomalous dataset of a Se-Methionine
16 (Se-Met) derived crystal was collected to 2.1 Å resolution at the peak of the Se-Met signal,
17 as measured by X-ray fluorescence, on ID29 (66) at the European Synchrotron Radiation
18 Facility (ESRF). For structural solution 8 Se-Met sites were located on the basis of their
19 anomalous differences using SHELXC/D/E (67). These sites were subsequently refined and
20 experimental phases were calculated using the single anomalous dispersion (SAD)
21 procedure in SHARP (68). These phases were further improved by density modification
22 followed by model building with the Buccaneer software (69). Model building and refinement
23 were performed using CCP4i suite program for crystallography (PHASER, ARP/wARP,
24 REFMAC5, COOT) (70-74).

25 For the RED_{mid}-SMU1_{Nter} complex, the crystals (native and Se-Met derivative) were obtained
26 in 0.1 M Hepes pH 7.0-7.5, 8-10 % PEG 8K and cryoprotected in the same solution + 30 %
27 glycerol. Data were processed with XDS and the structure was solved by molecular
28 replacement using the native SMU1_{Nter} structure. The model was built using the anomalous

1 signal of a Se-met derivative dataset for an accurate side chains attribution, and the final
2 refinement was performed using the software mentioned above.

3 For the SMU1_{Nter} + LSP641 complex, a mother solution of LSP641 was prepared at 125 mM
4 in DMSO. The co-crystals of SMU1_{Nter} (10 mg/mL) + 2.5 mM LSP641 were obtained in 0.1 M
5 Bis-Tris pH 6, 16-20 % PEG 10K, 0.2 M ammonium acetate and cryoprotected in the same
6 solution + 30 % glycerol. Data collection, processing and model building were performed as
7 described above.

8

9 ***In silico screening***

10 Docking experiments were performed using the LibDock protocol, as implemented in
11 Discovery Studio (Discovery Studio Modeling Environment, release 4.5; Dassault Systemes
12 BIOVIA: San Diego, 2015), an interface to the LibDock program developed by Diller and
13 Merz (75). LibDock uses protein site features referred to as HotSpots that fall into two
14 categories: polar and apolar HotSpots. The receptor HotSpot file was calculated prior to the
15 docking procedure. Random ligand conformations were generated from the initial ligand
16 structure through high-temperature molecular dynamics using the BEST algorithm before
17 docking. The rigid ligand poses were placed into the active site and HotSpots were matched
18 as triplets. The poses were pruned and a final optimization step was performed before the
19 poses were scored. Ligand hydrogens, which were removed during the docking process,
20 were added back to the ligand poses and optimized by minimization. The poses with the
21 highest LibDock scores were retained and clustered according to their binding mode.

22

23 ***RNA extraction and reverse transcription-quantitative PCR***

24 Total RNA and poly(A)⁺ RNAs were extracted subsequently using the RNeasy Mini Kit
25 (Qiagen) and Oligotex mRNA Mini kit (Qiagen) following the manufacturer's protocol.
26 Reverse transcription (RT) was performed on 5-10 ng of total mRNA (or poly(A)⁺ RNA
27 equivalent) using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific).
28 Quantitative real-time PCR was performed on 2 μ L of a 1/10 dilution of the RT reaction using

1 the Luminaris Color Probe qPCR Master Mix (Thermo Scientific) and a Light Cycler 480
2 (Roche). The levels of NS1/NS2 and M1/M2 mRNAs were determined and normalized with
3 respect to GAPDH mRNA levels using the protocol described in (23). The levels of
4 PB2/PB2-S1 mRNAs were determined by semi-quantitative RT-PCR as described in (34).

5

6 ***Data Availability Statement***

7 Structure coordinates and diffraction data are available on the Protein Data Bank
8 (<http://www.pdb.org>) under accession codes PDB: 6Q8F (SMU1_{Nter}), 6Q8I (RED_{mid-}
9 SMU1_{Nter}), and 6Q8J (SMU1_{Nter}-LSP641).

10

11

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18

19

1 References

- 2 1. Kaufmann SHE, Dorhoi A, Hotchkiss RS, & Bartenschlager R (2018) Host-directed
3 therapies for bacterial and viral infections. *Nat Rev Drug Discov* 17(1):35-56.
- 4 2. Zumla A, *et al.* (2016) Host-directed therapies for infectious diseases: current status,
5 recent progress, and future prospects. *Lancet Infect Dis* 16(4):e47-63.
- 6 3. Krammer F, *et al.* (2018) Influenza. *Nat Rev Dis Primers* 4(1):3.
- 7 4. Hurt AC (2014) The epidemiology and spread of drug resistant human influenza
8 viruses. *Curr Opin Virol* 8:22-29.
- 9 5. Lackenby A, *et al.* (2018) Global update on the susceptibility of human influenza
10 viruses to neuraminidase inhibitors and status of novel antivirals, 2016-2017. *Antiviral Res*
11 157:38-46.
- 12 6. Hurt AC, *et al.* (2012) Characteristics of a widespread community cluster of H275Y
13 oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. *J Infect Dis* 206(2):148-157.
- 14 7. Takashita E, *et al.* (2015) Characterization of a large cluster of influenza
15 A(H1N1)pdm09 viruses cross-resistant to oseltamivir and peramivir during the 2013-2014
16 influenza season in Japan. *Antimicrob Agents Chemother* 59(5):2607-2617.
- 17 8. Meijer A, *et al.* (2009) Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-
18 08 season. *Emerg Infect Dis* 15(4):552-560.
- 19 9. Bank C, *et al.* (2016) An experimental evaluation of drug-induced mutational
20 meltdown as an antiviral treatment strategy. *Evolution* 70(11):2470-2484.
- 21 10. Goldhill DH, *et al.* (2018) The mechanism of resistance to favipiravir in influenza. *Proc*
22 *Natl Acad Sci U S A* 115(45):11613-11618.
- 23 11. Hayden FG, *et al.* (2018) Baloxavir Marboxil for Uncomplicated Influenza in Adults
24 and Adolescents. *N Engl J Med* 379(10):913-923.
- 25 12. Koszalka P, Tilmanis D, & Hurt AC (2017) Influenza antivirals currently in late-phase
26 clinical trial. *Influenza Other Respir Viruses* 11(3):240-246.
- 27 13. McKimm-Breschkin JL, *et al.* (2018) Prevention and treatment of respiratory viral
28 infections: Presentations on antivirals, traditional therapies and host-directed interventions at
29 the 5th ISIRV Antiviral Group conference. *Antiviral Res* 149:118-142.
- 30 14. Ramos EL, *et al.* (2015) Efficacy and safety of treatment with an anti-m2e monoclonal
31 antibody in experimental human influenza. *J Infect Dis* 211(7):1038-1044.
- 32 15. Wollacott AM, *et al.* (2016) Safety and Upper Respiratory Pharmacokinetics of the
33 Hemagglutinin Stalk-Binding Antibody VIS410 Support Treatment and Prophylaxis Based on
34 Population Modeling of Seasonal Influenza A Outbreaks. *EBioMedicine* 5:147-155.
- 35 16. Byrn RA, *et al.* (2015) Preclinical activity of VX-787, a first-in-class, orally bioavailable
36 inhibitor of the influenza virus polymerase PB2 subunit. *Antimicrob Agents Chemother*
37 59(3):1569-1582.
- 38 17. Rossignol JF (2014) Nitazoxanide: a first-in-class broad-spectrum antiviral agent.
39 *Antiviral Res* 110:94-103.
- 40 18. Haffizulla J, *et al.* (2014) Effect of nitazoxanide in adults and adolescents with acute
41 uncomplicated influenza: a double-blind, randomised, placebo-controlled, phase 2b/3 trial.
42 *Lancet Infect Dis* 14(7):609-618.
- 43 19. Colombo RE, *et al.* (2016) A phase 1 randomized, double-blind, placebo-controlled,
44 crossover trial of DAS181 (Fludase(R)) in adult subjects with well-controlled asthma. *BMC*
45 *Infect Dis* 16:54.

- 1 20. Zenilman JM, *et al.* (2015) Phase 1 clinical trials of DAS181, an inhaled sialidase, in
2 healthy adults. *Antiviral Res* 123:114-119.
- 3 21. van de Wakker SI, Fischer MJE, & Oosting RS (2017) New drug-strategies to tackle
4 viral-host interactions for the treatment of influenza virus infections. *Eur J Pharmacol*
5 809:178-190.
- 6 22. Watanabe T & Kawaoka Y (2015) Influenza virus-host interactomes as a basis for
7 antiviral drug development. *Curr Opin Virol* 14:71-78.
- 8 23. Fournier G, *et al.* (2014) Recruitment of RED-SMU1 complex by Influenza A Virus
9 RNA polymerase to control Viral mRNA splicing. *PLoS Pathog* 10(6):e1004164.
- 10 24. Bessonov S, Anokhina M, Will CL, Urlaub H, & Luhrmann R (2008) Isolation of an
11 active step I spliceosome and composition of its RNP core. *Nature* 452(7189):846-850.
- 12 25. Chung T, Wang D, Kim CS, Yadegari R, & Larkins BA (2009) Plant SMU-1 and SMU-
13 2 homologues regulate pre-mRNA splicing and multiple aspects of development. *Plant*
14 *Physiol* 151(3):1498-1512.
- 15 26. Papasaikas P, Tejedor JR, Vigevani L, & Valcarcel J (2015) Functional splicing
16 network reveals extensive regulatory potential of the core spliceosomal machinery. *Mol Cell*
17 57(1):7-22.
- 18 27. Spartz AK, Herman RK, & Shaw JE (2004) SMU-2 and SMU-1, *Caenorhabditis*
19 *elegans* homologs of mammalian spliceosome-associated proteins RED and fSAP57, work
20 together to affect splice site choice. *Mol Cell Biol* 24(15):6811-6823.
- 21 28. Hegele A, *et al.* (2012) Dynamic protein-protein interaction wiring of the human
22 spliceosome. *Mol Cell* 45(4):567-580.
- 23 29. Ulrich AKC, Schulz JF, Kamprad A, Schutze T, & Wahl MC (2016) Structural Basis for
24 the Functional Coupling of the Alternative Splicing Factors Smu1 and RED. *Structure*
25 24(5):762-773.
- 26 30. Boxem M, *et al.* (2008) A protein domain-based interactome network for *C. elegans*
27 early embryogenesis. *Cell* 134(3):534-545.
- 28 31. Le Corre L, *et al.* (2010) Synthesis and biological evaluation of a triazole-based library
29 of pyrido[2,3-d]pyrimidines as FGFR3 tyrosine kinase inhibitors. *Org Biomol Chem*
30 8(9):2164-2173.
- 31 32. Matrosovich M, Matrosovich T, Carr J, Roberts NA, & Klenk HD (2003)
32 Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus
33 sensitivity to neuraminidase inhibitors. *J Virol* 77(15):8418-8425.
- 34 33. Seitz C, Frensing T, Hoper D, Kochs G, & Reichl U (2010) High yields of influenza A
35 virus in Madin-Darby canine kidney cells are promoted by an insufficient interferon-induced
36 antiviral state. *J Gen Virol* 91(Pt 7):1754-1763.
- 37 34. Yamayoshi S, Watanabe M, Goto H, & Kawaoka Y (2016) Identification of a Novel
38 Viral Protein Expressed from the PB2 Segment of Influenza A Virus. *J Virol* 90(1):444-456.
- 39 35. Bertram K, *et al.* (2017) Cryo-EM Structure of a Pre-catalytic Human Spliceosome
40 Primed for Activation. *Cell* 170(4):701-713 e711.
- 41 36. Arkin MR, Tang Y, & Wells JA (2014) Small-molecule inhibitors of protein-protein
42 interactions: progressing toward the reality. *Chem Biol* 21(9):1102-1114.
- 43 37. Scott DE, Bayly AR, Abell C, & Skidmore J (2016) Small molecules, big targets: drug
44 discovery faces the protein-protein interaction challenge. *Nat Rev Drug Discov* 15(8):533-
45 550.
- 46 38. Zhang B, Golding BT, & Hardcastle IR (2015) Small-molecule MDM2-p53 inhibitors:
47 recent advances. *Future Med Chem* 7(5):631-645.

- 1 39. Weydert C, De Rijck J, Christ F, & Debysers Z (2016) Targeting Virus-host Interactions
2 of HIV Replication. *Curr Top Med Chem* 16(10):1167-1190.
- 3 40. Fosgerau K & Hoffmann T (2015) Peptide therapeutics: current status and future
4 directions. *Drug Discov Today* 20(1):122-128.
- 5 41. Dassah M, Patzek S, Hunt VM, Medina PE, & Zahler AM (2009) A genetic screen for
6 suppressors of a mutated 5' splice site identifies factors associated with later steps of
7 spliceosome assembly. *Genetics* 182(3):725-734.
- 8 42. Kanno T, *et al.* (2017) A Genetic Screen for Pre-mRNA Splicing Mutants of
9 Arabidopsis thaliana Identifies Putative U1 snRNP Components RBM25 and PRP39a.
10 *Genetics* 207(4):1347-1359.
- 11 43. Verbist B, *et al.* (2015) Using transcriptomics to guide lead optimization in drug
12 discovery projects: Lessons learned from the QSTAR project. *Drug Discov Today* 20(5):505-
13 513.
- 14 44. Yeh PC, Yeh CC, Chen YC, & Juang YL (2012) RED, a spindle pole-associated
15 protein, is required for kinetochore localization of MAD1, mitotic progression, and activation
16 of the spindle assembly checkpoint. *The Journal of biological chemistry* 287(15):11704-
17 11716.
- 18 45. Ren L, *et al.* (2013) Loss of Smu1 function de-represses DNA replication and over-
19 activates ATR-dependent replication checkpoint. *Biochem Biophys Res Commun*
20 436(2):192-198.
- 21 46. Shah VJ & Maddika S (2018) CRL7(SMU1) E3 ligase complex-driven H2B
22 ubiquitylation functions in sister chromatid cohesion by regulating SMC1 expression. *J Cell*
23 *Sci* 131(8).
- 24 47. Jiang W, *et al.* (2013) Influenza A virus NS1 induces G0/G1 cell cycle arrest by
25 inhibiting the expression and activity of RhoA protein. *J Virol* 87(6):3039-3052.
- 26 48. Fan Y, *et al.* (2017) Cell Cycle-independent Role of Cyclin D3 in Host Restriction of
27 Influenza Virus Infection. *J Biol Chem* 292(12):5070-5088.
- 28 49. Teixeira VH, *et al.* (2013) Stochastic homeostasis in human airway epithelium is
29 achieved by neutral competition of basal cell progenitors. *Elife* 2:e00966.
- 30 50. Nikolaidis NM, *et al.* (2017) Mitogenic stimulation accelerates influenza-induced
31 mortality by increasing susceptibility of alveolar type II cells to infection. *Proc Natl Acad Sci U*
32 *S A* 114(32):E6613-E6622.
- 33 51. Kaida D, *et al.* (2007) Spliceostatin A targets SF3b and inhibits both splicing and
34 nuclear retention of pre-mRNA. *Nat Chem Biol* 3(9):576-583.
- 35 52. Kotake Y, *et al.* (2007) Splicing factor SF3b as a target of the antitumor natural
36 product pladienolide. *Nat Chem Biol* 3(9):570-575.
- 37 53. Fan L, Lagisetti C, Edwards CC, Webb TR, & Potter PM (2011) Sudemycins, novel
38 small molecule analogues of FR901464, induce alternative gene splicing. *ACS Chem Biol*
39 6(6):582-589.
- 40 54. Bonnal S, Vigevani L, & Valcarcel J (2012) The spliceosome as a target of novel
41 antitumor drugs. *Nat Rev Drug Discov* 11(11):847-859.
- 42 55. Effenberger KA, Urabe VK, & Jurica MS (2017) Modulating splicing with small
43 molecular inhibitors of the spliceosome. *Wiley Interdiscip Rev RNA* 8(2).
- 44 56. Hsu TY, *et al.* (2015) The spliceosome is a therapeutic vulnerability in MYC-driven
45 cancer. *Nature* 525(7569):384-388.
- 46 57. Lagisetti C, *et al.* (2009) Synthetic mRNA splicing modulator compounds with in vivo
47 antitumor activity. *J Med Chem* 52(22):6979-6990.

- 1 58. Lee SC, *et al.* (2016) Modulation of splicing catalysis for therapeutic targeting of
2 leukemia with mutations in genes encoding spliceosomal proteins. *Nat Med* 22(6):672-678.
- 3 59. Jager S, *et al.* (2012) Global landscape of HIV-human protein complexes. *Nature*
4 481(7381):365-370.
- 5 60. Konig R, *et al.* (2008) Global analysis of host-pathogen interactions that regulate
6 early-stage HIV-1 replication. *Cell* 135(1):49-60.
- 7 61. Rozenblatt-Rosen O, *et al.* (2012) Interpreting cancer genomes using systematic host
8 network perturbations by tumour virus proteins. *Nature* 487(7408):491-495.
- 9 62. Lee SC & Abdel-Wahab O (2016) Therapeutic targeting of splicing in cancer. *Nat Med*
10 22(9):976-986.
- 11 63. Salton M & Misteli T (2016) Small Molecule Modulators of Pre-mRNA Splicing in
12 Cancer Therapy. *Trends Mol Med* 22(1):28-37.
- 13 64. Cassonnet P, *et al.* (2011) Benchmarking a luciferase complementation assay for
14 detecting protein complexes. *Nat Methods* 8(12):990-992.
- 15 65. Kabsch W (2010) Integration, scaling, space-group assignment and post-refinement.
16 *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):133-144.
- 17 66. de Sanctis D, *et al.* (2012) ID29: a high-intensity highly automated ESRF beamline for
18 macromolecular crystallography experiments exploiting anomalous scattering. *J Synchrotron*
19 *Radiat* 19(Pt 3):455-461.
- 20 67. Sheldrick GM (2010) Experimental phasing with SHELXC/D/E: combining chain
21 tracing with density modification. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):479-485.
- 22 68. de La Fortelle E & Bricogne G (1997) [27] Maximum-likelihood heavy-atom parameter
23 refinement for multiple isomorphous replacement and multiwavelength anomalous diffraction
24 methods. *Methods Enzymol* 276:472-494.
- 25 69. Cowtan K (2006) The Buccaneer software for automated model building. 1. Tracing
26 protein chains. *Acta Crystallogr D Biol Crystallogr* 62(Pt 9):1002-1011.
- 27 70. Cohen SX, *et al.* (2008) ARP/wARP and molecular replacement: the next generation.
28 *Acta Crystallogr D Biol Crystallogr* 64(Pt 1):49-60.
- 29 71. Emsley P & Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta*
30 *Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2126-2132.
- 31 72. McCoy AJ, *et al.* (2007) Phaser crystallographic software. *J Appl Crystallogr* 40(Pt
32 4):658-674.
- 33 73. Murshudov GN, Vagin AA, & Dodson EJ (1997) Refinement of macromolecular
34 structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53(Pt
35 3):240-255.
- 36 74. Winn MD, *et al.* (2011) Overview of the CCP4 suite and current developments. *Acta*
37 *Crystallogr D Biol Crystallogr* 67(Pt 4):235-242.
- 38 75. Diller DJ & Merz KM, Jr. (2001) High throughput docking for library design and library
39 prioritization. *Proteins* 43(2):113-124.
- 40 76. The PyMOL Molecular Graphics System Version 2.0 Schrödinger LLC.

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42

1 **Figure legends**

2

3 **Figure 1. RED-SMU1 interaction mapping using a cell-based assay.**

4 The *Gaussia princeps* luciferase-based complementation assay was performed as described
5 in the Methods section. Gluc2-fused RED full-length (RED) or indicated subdomains were
6 coexpressed with Gluc1-fused SMU1 full-length (SMU1) or SMU1[1-196] (SMU1_{Nter}) by
7 transient transfection in HEK-293T cells. **A.** The normalized luciferase activities are
8 expressed as percentages relative to the activity measured with full-length RED and SMU1
9 proteins. The data shown are the mean +/- SD of three independent experiments in
10 triplicates. Black and grey bars represent luciferase activities measured in the presence of
11 SMU1 and SMU1_{Nter}, respectively. ** : p<0.01 (parametric unpaired t-test). On the schematic
12 diagram of RED its characteristic stretch of repeated arginine, glutamic acid and aspartic
13 acid residues is represented by a hatched box. The dotted line highlights the interaction
14 between the two components of the minimal SMU1_{Nter}-RED_{mid} complex. **B.** Cell lysates used
15 to determine luciferase activities in A were subsequently analysed by western blot using
16 antibodies specific for *Gaussia princeps* luciferase (Gluc, upper panel) and Glyceraldehyde
17 phosphate deshydrogenase (GAPDH, lower panel). White arrowhead: Gluc1-SMU1; Open
18 arrowhead: Gluc2-RED; Black arrowhead: Gluc2-RED[206-260], renamed Glu2-RED_{mid} in the
19 text.

20

21 **Figure 2: Crystal structure of the recombinant human RED_{mid}-SMU1_{Nter} complex.**

22 **A.** Global view of the complex. The RED_{mid} and SMU1_{Nter} proteins are coloured in red and
23 yellow respectively. One monomer of SMU1_{Nter} is shown as a ribbon diagram and the second
24 as a surface representation. **B.** Close-up view of the mixed β -sheet formed by the interaction
25 of RED[235-257] with the N-terminal β -strand of a SMU1_{Nter} monomer, and stabilized by the
26 C-terminal surface of the other dimer partner SMU1_{Nter} molecule. **C.** Details of the second
27 interface corresponding to the α -helical part of RED_{mid} inserted into a hydrophobic groove at
28 the surface of SMU1_{Nter}. The images were generated using PyMOL (76).

1 **Figure 3. Structure-based analysis of the RED-SMU1 interface.**

2 **A.** Schematic representation of Interface II. On the helical wheel representation of RED
3 (residues 213 to 222), aliphatic residues (in a grey frame) are concentrated at the RED-
4 SMU1 interface. The polar residues (in blue) are exclusively located on the opposite side. **B.**
5 Location of SMU1 and RED residues (coloured in green and purple, respectively) involved in
6 interactions at interface II and subjected to mutagenesis experiments shown in panels C to
7 H. **C-E.** Luciferase-based complementation assays were performed as described in the
8 Methods section with the indicated combinations of wild-type (wt) or mutant proteins. The
9 normalized luciferase activities are expressed as percentages relative to the activity
10 measured in the presence of the wt SMU1 and RED proteins. The data shown are the mean
11 +/- SD of three independent experiments in triplicates, except when SMU1-D57A-E89A was
12 tested in (C): two independent experiments. ** p<0.01, *** p<0.001 unpaired t-test. **F.** The
13 indicated combinations of wt or mutant SMU1 and RED proteins, fused to the Strep-tag, were
14 transiently co-expressed in HEK-293T cells. Cell lysates were analysed by western blot,
15 using HRP-conjugated Streptactin (lower panel). GAPDH (upper panel) was used as a
16 loading control. **G-H.** Cell lysates used to determine luciferase activities in C and D were
17 subsequently analysed by western blot, using antibodies specific for Gluc (lower panel) and
18 an anti-GAPDH antibody (upper panel).

19

20 **Figure 4. SMU1_{Nter} binding to compounds LSP641 and LSP61.**

21 **A.** Flow chart of *in silico* screening and compound evaluation. The indicated assays were
22 performed as described in the Methods section, in the presence of compounds at 60 μ M or
23 DMSO. The Venn diagrams show the number of selected compounds. The cut-off applied
24 are indicated in italics (cell viability assay: < 2-fold reduction in CellTiter-Glo signal after 36h
25 incubation with the compound compared to DMSO; RED-SMU1 and IAV replication assays:
26 $\geq 20\%$ and $\geq 80\%$ reduction in Gaussia luciferase and Nanoluc signal, respectively, in the
27 presence of the compound compared to DMSO). **B.** SMU1_{Nter} residues required for efficient
28 binding to RED (coloured in green) and used for filtering of the docking poses. **C.** Chemical

1 structures of compounds LSP641 and LSP61. **D.** Co-crystal structure of LSP641 in complex
2 with SMU1_{Nter}. The LSP641 binding pocket is coloured according to hydrophobicity (blue:
3 hydrophilic, red: hydrophobic). **E.** Residues of SMU1 involved in LSP641 binding. Green: key
4 residues for RED binding which also interact with LSP641. Orange: other residues which
5 strongly interact with LSP641 in the co-crystal structure. **F.** Representative pose of LSP61
6 upon *in silico* docking on SMU_{Nter}. The LSP61 binding pocket is coloured according to
7 hydrophobicity (blue: hydrophilic, red: hydrophobic). **G.** Residues of SMU1 predictively
8 involved in LSP61 binding. Green: key residues for RED binding which also interact with
9 LSP61 upon molecular docking. Orange: other residues which strongly interact with LSP61
10 upon molecular docking.

11

12 **Figure 5. Evaluation of compound LSP61 for RED-SMU1 destabilisation.**

13 **A.** RED-SMU1 interaction assay. The split-luciferase assay was performed as described in
14 the Methods section. At 8 hours post-transfection (hpt), the A549 cells were incubated with
15 the relevant compounds at the concentrations indicated. The normalized luciferase units
16 measured at 24 hpt are expressed as percentages relative to the DMSO-treated control. The
17 data shown are the mean +/- SD of three independent experiments in triplicates. ** p<0.005
18 (parametric unpaired t-test). **B.** The split luciferase interaction assay was performed as in A,
19 using Gluc1-FOS and Gluc2-JUN plasmids. The data shown are the mean +/- SD of two
20 independent experiments in triplicates. **C.** Effect on the steady-state levels of the
21 endogenous RED and SMU1 proteins. A549 cells were incubated with the indicated
22 concentrations of LSP61 (+) or with DMSO (-) for 24h. Total cell extracts were analyzed by
23 western blot, using antibodies specific for RED, SMU1 and GAPDH. **D.** Cell viability assay.
24 A549 cells were incubated with the indicated concentrations of LSP61 or with DMSO. ATP
25 levels, which reflect the number of viable cells, were determined using the CellTiter-Glo kit
26 (Promega) at the onset of the experiment (T0), and following 24 or 48 hours of incubation
27 (T24, T48). The data shown are the mean of luciferase units +/- SD of two independent
28 experiments in duplicates.

1 **Figure 6. Evaluation of compound LSP61 for anti-IAV activity.**

2 **A.** Effect on IAV replication. A549 cells were infected with the WSN-Nanoluc or RSV-Firefly
3 virus (0.001 and 0.01 PFU/cell, respectively) and incubated with LSP641 or LSP61 at the
4 indicated concentrations, or with DMSO. The luciferase units measured at 24 hpi are
5 expressed as percentages relative to DMSO-treated control. The data shown are the mean
6 +/- SD of three (WSN-Nanoluc) or two (RSV-luc) independent experiments in triplicates.
7 **p<0.005, ***p<0.0001 (parametric paired t-test). **B-C.** Effect on the production of infectious
8 particles. MDCK-SIAT (B) or A549 cells (C) were infected with the WSN, H1N1pdm09 or
9 H3N2 viruses as indicated, and incubated with 60 µM of LSP61 or with DMSO. At 24, 48,
10 and 72 hpi, the supernatants were collected and viral titers determined by plaque assay. The
11 data are expressed as mean +/- SD of three independent experiments (except for the 24 hpi
12 time-point in C and D : two independent experiments), each in triplicates that were pooled for
13 titration. * p<0.05, ** p<0.01, *** p<0.001, parametric paired t-test. **D.** Effect on NS1 mRNA
14 splicing. A549 cells were infected with the WSN-wt virus (5 PFU/cell), and incubated with 60
15 µM of LSP641 or LSP61, or with DMSO. After 6 h incubation, polyA+ RNA were extracted
16 and the levels of NS2 and NS1 mRNAs were analyzed by quantitative real-time PCR and
17 normalized with respect to GAPDH mRNA levels. The NS2/NS1 mRNA ratios shown are the
18 mean +/- SD of three independent experiments in duplicates. p=0.01, parametric paired t-
19 test.

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Figure 1

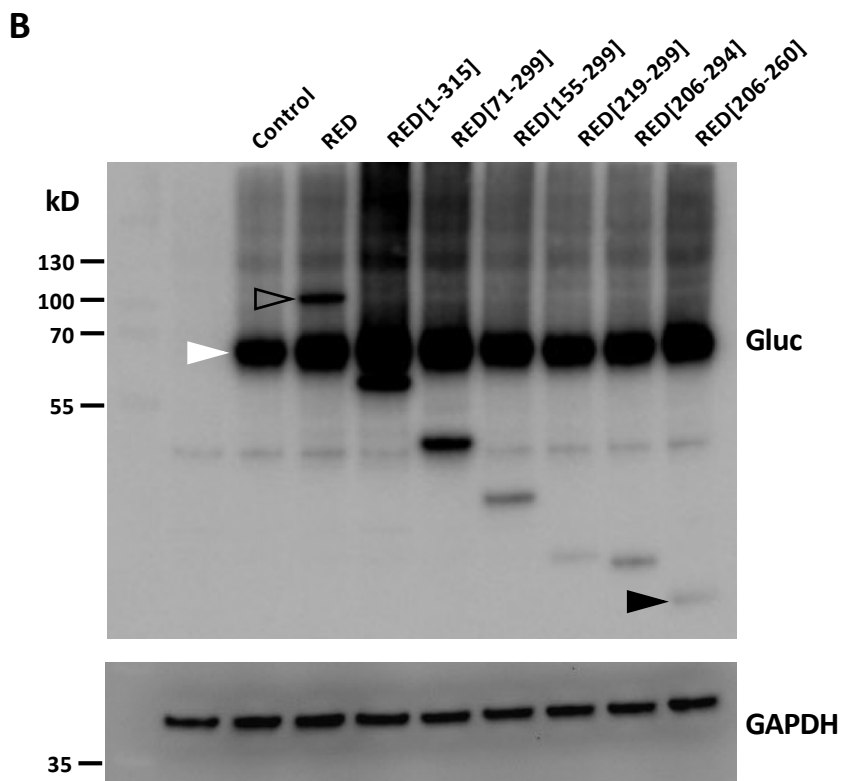
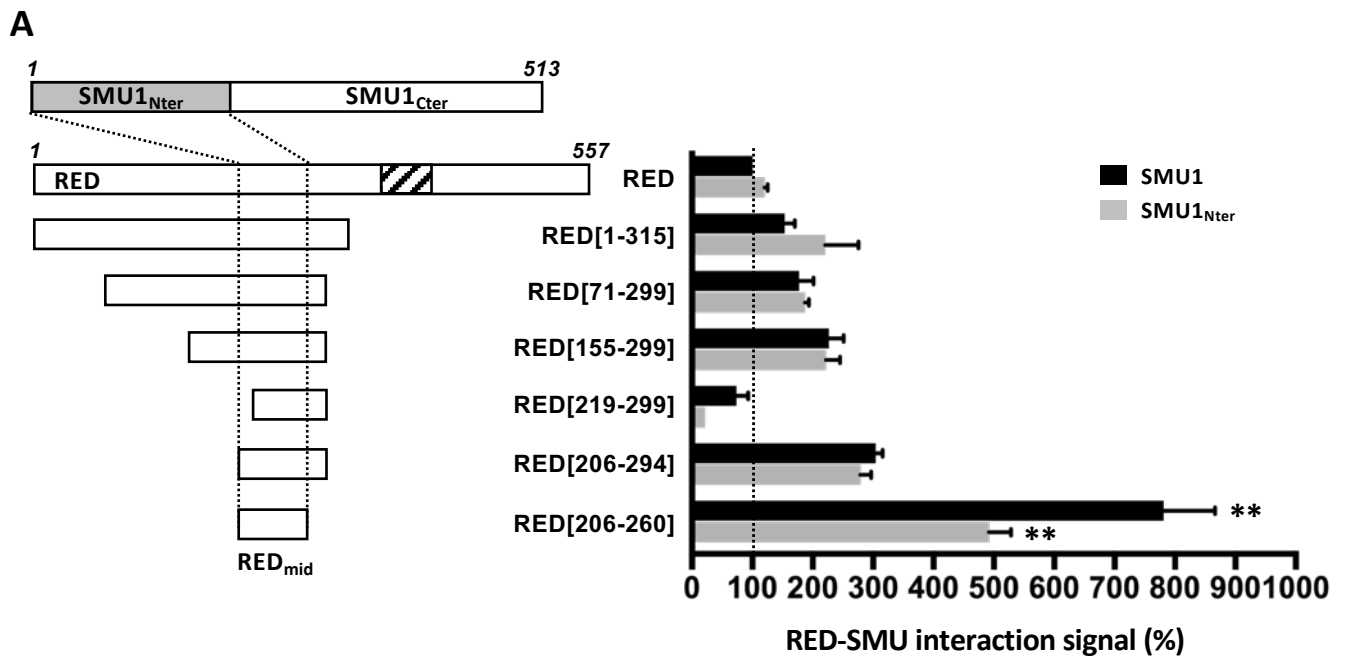


Figure 2

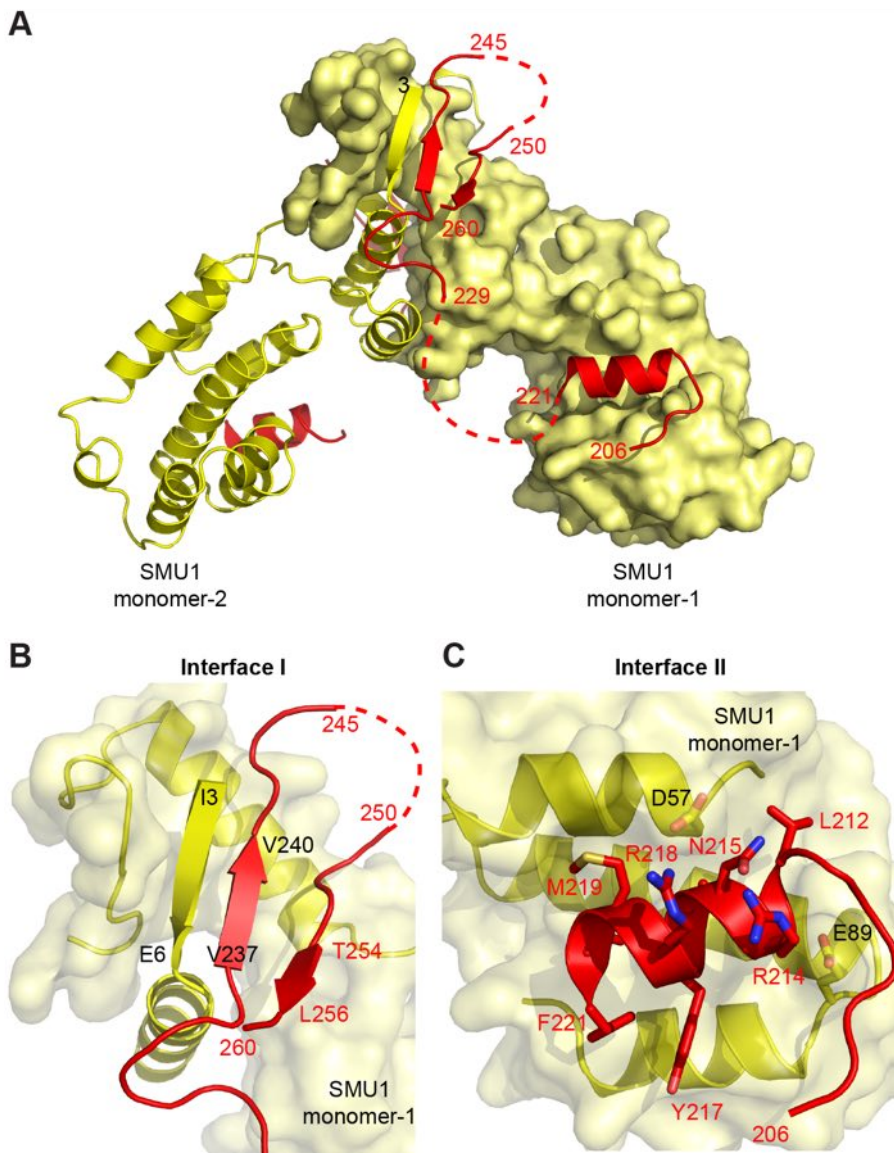


Figure 3

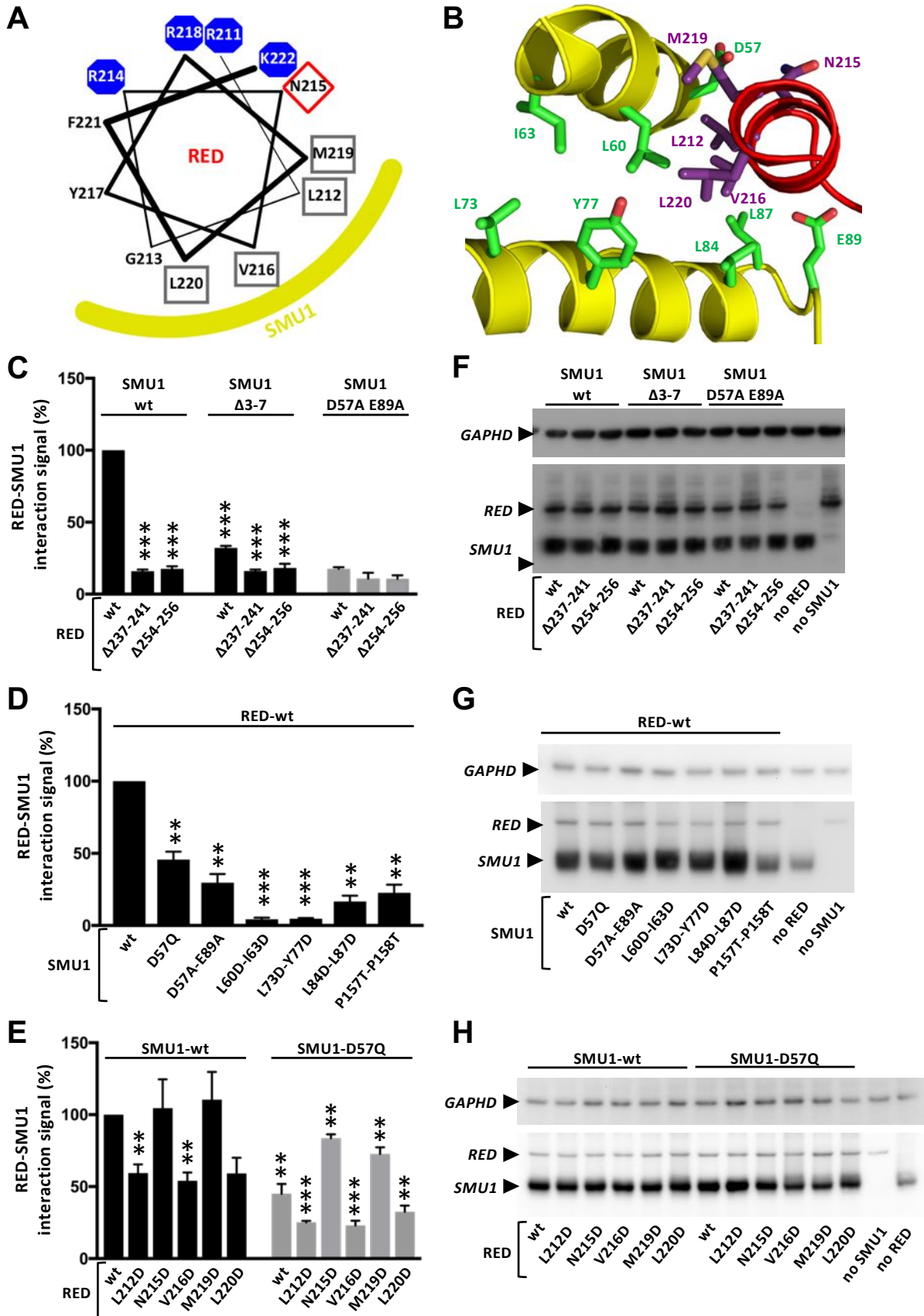


Figure 4

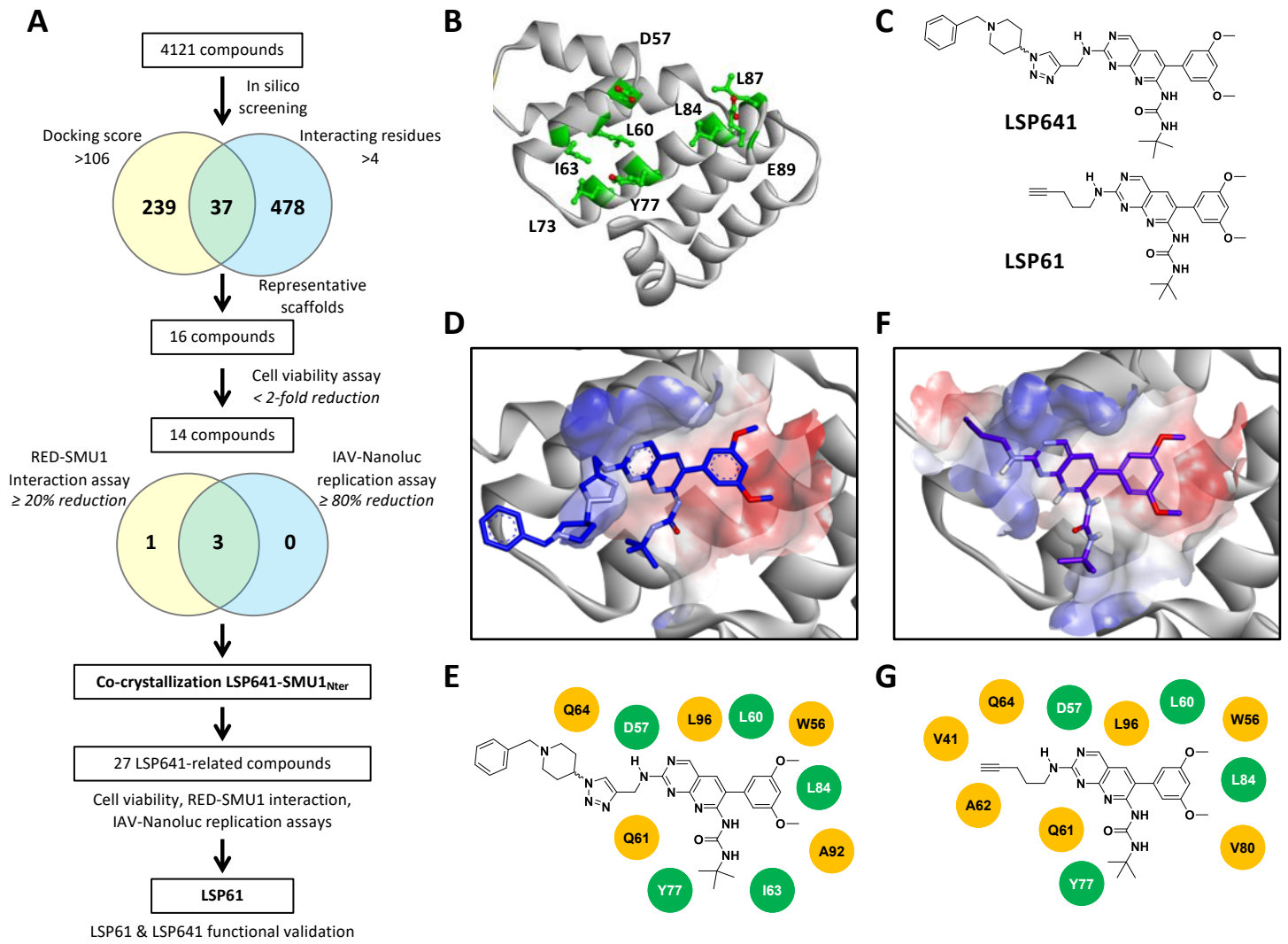


Figure 5

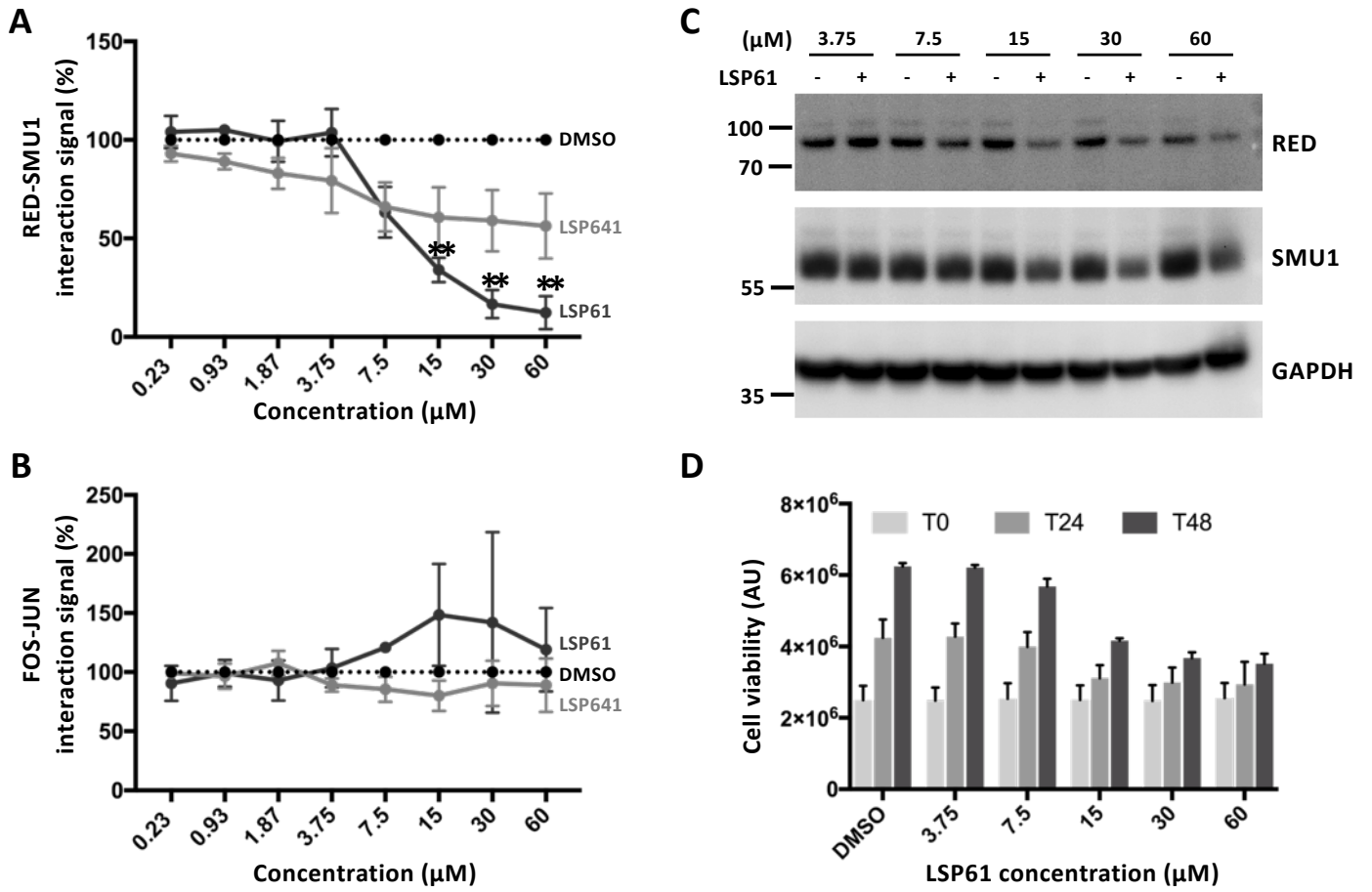


Figure 6

