Ebola and Marburg virus VP35 coiled-coil validated as antiviral target by tripartite split-GFP complementation

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Highlights

- Ebola and Marburg virus VP35 oligomerize via trimeric and tetrameric coiled-coil
- VP35 coiled-coil assembly triggers fluorescence of a tripartite split-GFP system
- Mutations perturbing VP35 coiled-coil assembly hamper split-GFP complementation
- Myricetin and TBBT inhibit split-GFP complementation mediated by VP35 coiled-coil

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Ebola and Marburg virus VP35 coiled-coil validated as antiviral target by tripartite split-GFP complementation

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SUMMARY

Ebola virus (EBOV) and Marburg virus (MARV) are highly pathogenic viruses in humans, against which approved antivirals are lacking. During EBOV and MARV infection, coiled-coil mediated oligomerization is essential for the virion protein 35 (VP35) polymerase co-factor function and type I interferon antagonism, making VP35 coiled-coil an elective drug target. We established a tripartite split-green fluorescent protein (GFP) fluorescence complementation (FC) system based on recombinant GFP-tagged EBOV and MARV VP35, which probes VP35 coiled-coil assembly by monitoring fluorescence on E. coli colonies, or in vitro in 96/384-multiwell. Oligomerization-defective VP35 mutants showed that correct coiled-coil knobs-into-holes pairing within VP35 oligomer is pre-requisite for GFP tags and GFP detector to reconstitute fluorescing full-length GFP. The method was validated by screening a small compound library, which identified Myricetin and 4,5,6,7-Tetrabromobenzotriazole as inhibitors of EBOV and MARV VP35 oligomerization-dependent FC with low-micromolar IC$_{50}$ values. These findings substantiate the VP35 coiled-coil value as antiviral target.

INTRODUCTION

Ebola virus (EBOV, Zaire ebolavirus species) and Marburg virus (MARV, Marburg marburgvirus species) are the two prototypical members of the Filoviridae family of the order Mononegavirales, which comprises six recognized genera (namely Cuevavirus, Dianlovirus, Ebolavirus, Marburgvirus, Striavirus, and Thamnovirus) whose species possess a characteristic filamentous-shaped virion and genetic organization of single-stranded, non-segmented, negative-sensed RNA genome (Kuhn et al., 2019). EBOV and MARV are highly pathogenic in humans and non-human primates, causing an often fatal disease with up to 90% lethality. While having been sporadic over the past decades and confined to remote rural areas of the Sub-Saharan Africa, EBOV and MARV human outbreaks have in recent years increased in frequency and number of cases, expanded geographically and extended in duration (Languon and Quaye, 2019). Exceptional efforts have been made in developing therapeutic countermeasures against filoviruses, which recently led to the approval of two EBOV vaccines and a human monoclonal antibody cocktail. However, in spite of the promising results shown by several compound candidates, no small molecule drugs have gained licensing to date (Hansen et al., 2021). This urges for keeping up the pursuit of novel therapeutics on the one hand and for the development of biochemical tools to explore the antiviral target potential of functional domains in filoviral proteins on the other hand (Edwards and Basler, 2019). One of the key aspects that correlates with the high lethality of EBOV and MARV infection is the coupling of viral replication, that proceeds unarrested, together with the potent suppression of the host innate immune response (Olejnik et al., 2017). Among the nine proteins encoded by the filoviral genome, one in particular - the virion protein 35 (VP35) - is involved in both those processes. VP35 is the ortholog of the phosphoprotein (P) of the other viruses in the Mononegavirales. It is a modular protein of 340 and 329 amino acids in length in EBOV and MARV, respectively (Figure 1A), whose domain structures have been solved in recent years, thus providing the structural basis to dissect and elucidate its multifunctional nature. In the context of filovirus replication, where VP35 acts as indispensable co-factor for the viral RNA-dependent RNA polymerase (RdRp) large (L) protein, the region responsible for interaction with L is deemed to be located within the central coiled-coil of the oligomerization domain, and VP35 is thought to tether the L polymerase to the ribonucleoprotein (RNP) complex, forming a scaffold for viral replication.
Figure 1. EBOV and MARV VP35 oligomerization domains tripartite split-GFP FC system
(A) Structural organization of EBOV and MARV VP35 proteins; the position of the VP35 gene in the filoviral genome and the oligomerization domain in the protein of the two filoviral species are highlighted in dark gray and avocado and maroon color, respectively.

(B) Cartoon model illustrating the split of the full-length eGFP into the three components 9M6, 10M2, 11M4 (colored in laurel, dark lime and olive, respectively), and the design of the GFP 10M2-fused EBOV (residues 75–185), MARV (residues 58–174) VP35 oligomerization domains and GFP 9M6 (residues 1–196) recombinant constructs (MW, molecular weight).

(C) Cartoon model illustrating the heterologous expression in E. coli cells of recombinant VP35 oligomerization domain fused to split-GFP 10M2 and 11M4 tags, and of GFP 9M6, in various combinations for the single expression, double and triple co-expression of the chimeric proteins.
RNA synthesis (Bruhn et al., 2017; Ramaswamy et al., 2018; Zinzula et al., 2019; Chanthamontri et al., 2019). VP35 also binds to the viral nucleoprotein (NP) via its unstructured N-terminal region, thereby preventing NP protomers from unspecific coating of host and viral RNA, and chaperoning them to the de novo-synthesized viral genome for proper assembly of nascent RNP complexes (Leung et al., 2015; Kirchdoerfer et al., 2015; Zhu et al., 2017; Landeras-Bueno et al., 2019). With respect to innate immunity evasion, VP35 interacts with dsRNA byproducts of viral replication via its C-terminal dsRNA binding/interferon (IFN)-inhibitory domain (RBD/IID). By doing so, VP35 impedes that viral nucleic acids are recognized as pathogen-associated molecular patterns (PAMPs) by cytoplasmic RNA helicases of the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) family, ultimately suppressing induction of type I interferon (IFN) (Leung et al., 2009, 2010a, 2010b; Kimberlin et al., 2010; Bale et al., 2012, 2013; Ramanan et al., 2012; Zinzula et al., 2012). To function as replicative co-factor as well as IFN antagonist, VP35 is required to be in oligomeric form. In fact, alanine mutations of highly conserved hydrophobic residues within the VP35 oligomerization domain (Leu90, Leu93 and Leu107 in EBOV; Leu90 and Leu104 in MARV) disrupt the coiled-coil, thereby abolishing viral replication and potently diminishing VP35 IFN inhibition capability (Reid et al., 2005; Möller et al., 2005). Crystal structures and biophysical in-solution data revealed a trimeric organization of the isolated VP35 oligomerization domain for MARV, trimeric and tetrameric forms for EBOV, and exclusively tetrameric assemblies for all the other ebolaviral species (Bruhn et al., 2017; Zinzula et al., 2019). In both the trimeric and tetrameric states, oligomerization is mediated by a canonical coiled-coil motif consisting of five or four amino acid heptad repeats in EBOV and MARV, respectively. Moreover, residues whose mutation were found to be detrimental to viral replication and immune evasion are located at positions a and d of the abcdedf heptad register, pointing towards the coiled-coil axis to form its inner hydrophobic core (Zinzula et al., 2019). Elucidation of the structural basis for such defective phenotypes has thus reinforced the importance of VP35 function for determinacy of EBOV and MARV virulence and pathogenesis. Furthermore, these mutations have highlighted the potential and attractiveness of the VP35 coiled-coil oligomerization domain as a unique antiviral target. However, no biochemical assay has been established thus far to monitor VP35 oligomerization in vitro, and no readout to probe this process for antiviral screening purposes has been available. Previous studies on full-length recombinant constructs showed that no VP35 monomeric species exist in solution (Zinzula et al., 2009; Kimberlin et al., 2010; Bruhn et al., 2017; Ramaswamy et al., 2018). Nonetheless, biophysical characterization of isolated VP35 oligomerization domains revealed that the coiled-coil-mediated quaternary structures are highly stable and not subject to a dynamic equilibrium at physiological conditions (Bruhn et al., 2017; Zinzula et al., 2019). Considering that VP35 oligomers presumably form immediately after protein translation, these features limit the investigation on VP35 assemblies to techniques that can detect oligomerization ex-post facto. In recent years, a new experimental approach to probe protein-protein interactions has been developed that relies on a tripartite split-green fluorescent protein (GFP) system whose fragments are fused as GFP tags to protein partners. Only as a direct consequence of oligomer formation of the protein partners, the GFP tags can properly orient and mutually interact, then bind to a third GFP fragment that serves as reporter sensor. Hence, upon assembly of the tripartite split-GFP, the full-length GFP protein is reconstituted into a functional fluorophore, which is termed fluorescence complementation (FC) (Cabantous et al., 2013). The technique was proven to efficiently detect protein-protein interactions in mammalian and E. coli cells, with very low fluorescence background and lack of false-positives that because of the spontaneous self-association of GFP-tags even in the absence of complex formation between the tagged partners, occasionally arise in conventional bimolecular FC (Koraič et al., 2018). The central α-helical region of the VP35 oligomerization domain is the one bearing the coiled-coil motif that self-associates in parallel orientation to build homo-trimeric or homo-tetrameric assemblies (Bruhn et al., 2017; Zinzula et al., 2019). The vicinity between α-helices in both oligomeric forms, the intrinsic high stability of their coiled-coil and the high degree of solubility of this domain compared to the full-length protein, render the VP35 oligomerization domain particularly suitable for the placement of small non-perturbing fusion tags at its boundaries. Therefore, we exploited the properties of the tripartite split-GFP system by engineering the EBOV and MARV VP35 oligomerization domains, which we used to establish a novel biochemical assay that makes VP35 oligomerization accessible to inhibitor screening.

Figure 1. Continued

(D) Blue Coomassie-stained SDS-PAGE analysis of IMAC and SEC purified recombinant EBOV VP3575-185 and MARV VP3558-174 oligomerization domains (in their native non-tagged and GFP-tagged versions, respectively) and of IMAC and SEC purified recombinant GFP 9M6, oligomeric protein bands resistant to full denaturation by heat, reducing agents and SDS, are visible in most VP35 samples as migrating at ~ 70 kDa; faint bands reminiscent of protein degradation products are visible in most samples as migrating below the monomeric band of the constructs. (M, molecular weight marker).
RESULTS AND DISCUSSION

Design of a tripartite split-GFP FC system to probe EBOV and MARV VP35 oligomerization

Recombinant EBOV (residues 75–185) and MARV (residues 58–174) VP35 constructs (Figure 1A) were designed for bacterial expression, each N-terminally fused with either GFP β-strand 10M2 (residues 197–215) or GFP β-strand 11M4 (residues 216–237) tags of the enhanced GFP (eGFP, residues 1–237) (Figures 1B, 1C, 1S1, and 1S2). These constructs are longer versions of the VP35 oligomerization domain as compared to previous ones (Bruhn et al., 2017; Zinzula et al., 2019), containing an amino acid tract upstream to the canonical coiled coil and downstream its adjacent highly conserved amphipathic chain, the latter of which was recently found capable of nucleoside triphosphatase (NTPase)/helicase activity (Shu et al., 2019).

Also, a recombinant GFP truncation derived from the previously described GFP 1–9 OPT fragment (residues 1–193) (Cabantous et al., 2013) and here named as GFP 9M6 (residues 1–196), was designed to function as detector component in the tripartite system (Figures 1B and 1S1). Recombinant proteins bearing an N-terminal (for GFP 9M6) or C-terminal (for GFP 10M2- and GFP 11M4-fused VP35) octahistidine (His8) tag were all expressed in E. coli, singly or co-expressed in all the combinations of two and three components (Figures 1B, 1C, and 1S2), then purified by immobilized metal-ion affinity chromatography (IMAC) and by size exclusion chromatography (SEC) followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 1D). Fractions corresponding to the main SEC peak of co-expressed GFP 10M2/11M4 VP35 oligomerization domains were pooled, then subjected to comparative biophysical characterization with respect to the native, non GFP-tagged VP35 constructs added of a hexahistidine (His6) tag at their C-termini, which were taken as controls (Figure S3). Analysis of the in solution oligomeric state by SEC coupled to multi-angle light scattering (SEC-MALS) showed that GFP 10M2/11M4 EBOV VP35 and non GFP-tagged EBOV VP35 oligomerization domains eluted each as major peak with average molecular weight (MW) of about 61.1 and 51.1 kDa, respectively (Figure 2A). Similarly, GFP 10M2/11M4 MARV VP35 and non-GFP tagged MARV VP35 oligomerization domains eluted as major peak with average MW of ~61.3 and ~53.8 kDa, respectively (Figure 2B). Considering the predicted monomeric size of ~17.2 kDa (GFP 10M2/11M4-tagged, average) and ~13.3 kDa (non GFP-tagged) for the EBOV construct, and of ~18.1 kDa (GFP 10M2/11M4-tagged, average) and ~14.3 kDa (non GFP-tagged) for the MARV one, these values correspond to 3.7–3.8 and 3.4–3.7-folds the EBOV and MARV VP35 oligomerization domain monomer, respectively. Hence, they are consistent with a mixed population of homo-trimers and homo-tetramers in solution, in agreement to what previously observed for filoviral VP35 oligomers (Zinzula et al., 2019).
Importantly, considering the plasticity displayed by filoviral VP35 coiled coils, which were previously shown to assemble into different, however nearly-isoenergetic oligomeric states (Ramaswamy et al., 2018; Zinzula et al., 2019; Di Palma et al., 2019), it is worth noting that the designed tripartite FC system is fully compatible with the formation of both VP35 homo-trimers and homo-tetramers. In fact, assuming no variation in the expression rate between the two GFP-tagged VP35 monomers (i.e., fused to GFP 10M2 or GFP 11M4), the different assortments of the two tags for a given VP35 oligomer are in principle equiprobable and, regardless whether the resulting oligomer is a homo-trimer or a homo-tetramer, the presence of two GFP tags represents the minimum requirement for the FC signal to occur (Figure 5A).

**Functional GFP is reconstituted upon EBOV and MARV VP35 oligomerization in vivo and in vitro**

Next, we wanted to evaluate whether the reconstitution of the GFP ternary complex, and therefore the FC, would occur in vivo and could be monitored through the rapid screening of fluorescent E. coli cell colonies. To this aim, agar plates seeded with triple transformants - concomitantly expressing GFP 9M6, GFP 10M2 VP35 and GFP 11M4 VP35 were compared to those with double transformants in various combinations and with single transformants. Only the co-expression of all components of the tripartite system – namely the VP35 oligomerization domains fused to the GFP 10M2 and 11M4 tags and the GFP 9M6 reporter – led to the growth of bright and fluorescent bacterial colonies, whereas negligible fluorescence comparable to the background was observed when one or more of the components were missing (Figure 3A). In parallel, the in vitro reconstitution of the GFP ternary complex by mixing purified EBOV and MARV GFP 10M2/11M4 VP35 with GFP 9M6 was assessed. To this aim, reactions were set up in 50–100 μL volume replicates on 96- or 384-well plates under close-to-physiological conditions (25 mM Tris pH 7.4, 100 mM NaCl), incubated for 1 h to allow the tethering of GFP 10M2/11M4 to GFP 9M6, followed by detection of the fluorescent signal. For both EBOV and MARV VP35, only the oligomers bearing both types of GFP tags, formed upon co-expression of GFP 10M2 and GFP 11M4-fused VP35, could reconstitute a full-length functional GFP when mixed after purification with an excess of the truncated GFP 9M6 reporter (Figure 3B). Moreover, complexes containing all three components showed fluorescence similar to intact GFP (Figure 3B, bars FCtrans VP35 WT). By contrast, as in the agar-plate screening, controls missing one or more components exhibited very little or no fluorescence. Of note, the ability to reconstitute a functional chromophore could not be fully restored when VP35 bearing the missing GFP-tag was provided in trans by adding the corresponding purified protein to the reaction (Figure 3B, bars FCtrans VP35 WT). On the one hand, this is a confirmation of the previous observation that once formed VP35 oligomers do not undergo dynamic dissociation and re-assembly (Zinzula et al., 2019). On the other hand, this rules out the possibility that two complementary GFP tags, remained uncoupled in their respective VP35 homo-trimers, may interact with GFP 9M6 and reconstitute a “bridged” full-length GFP, thereby leading to false positive read-outs that do not represent VP35 oligomerization-dependent FC events. Instead, results demonstrate that FC of the tripartite split-GFP, in vivo as well as in vitro, directly correlates with the preemptive formation of EBOV and MARV VP35 oligomers bearing an assortment of the two different GFP tags 10M2 and 11M4.

**Optimal biochemical parameters to monitor EBOV and MARV VP35 oligomerization by GFP FC**

Probing the EBOV and MARV VP35 oligomerization in a quantitative and reproducible manner required our assay to be robust, sensitive and also fast to be suitable for screening. Moreover, one peculiarity of adopting the tripartite split-GFP system to study this process is that, in contrast to other methods, protein-protein complex formation can be detected in this case only in retrospect. In addition, the ratio between the two types of tags GFP 10M2 and GFP 11M4 in a given VP35 oligomer is not tunable, because
it derives from stochastic assortment. Hence, as long as the presence of both 10M2 and 11M4 tags is ensured by co-expression of the respective GFP-tagged VP35 species, the amount of GFP 9M6 detector represents the only limiting reagent for FC to occur. We therefore aimed to define to what minimum excess, for how long and in which optimal biochemical conditions the detector component should be added to saturate the system for the best signal-to-noise ratio. We characterized the optimal assay parameters in terms of molar ratio between the GFP 9M6 detector and the GFP 10M2/11M4-fused VP35 oligomer, as well as in terms of incubation time and temperature. A titration experiment was conducted against a fixed molar amount (≈2.3 μM) of the co-expressed GFP 10M2/11M4 EBOV (or MARV) VP35 oligomerization domains, by varying the GFP 9M6 concentration by two-fold serial dilutions between 36.8 and 0.575 μM. In both EBOV and MARV samples, fluorescence steeply increased with increasing GFP 9M6 concentration up to a 4:1 M ratio between GFP 9M6 and GFP 10M2/11M4-tagged VP35, after which the fluorescence yield began to level off. (Figure 4A). Conversely, although the signal provided by the intrinsic fluorescence of the GFP 9M6 negative control increased linearly and proportionally to its molar amount, intensity remained close to background level even at the highest concentration tested (Figure 4A). Fluorescence was also measured at a 4:1 ratio over an incubation period of 15–240 min. Signal intensity steeply increased up to 60 min and gradually leveled off toward 240 min (Figure 4B). Furthermore, the fluorescence at three different incubation temperatures (4, 23 and 37°C) was recorded, showing no significant variation for the EBOV VP35 construct, whereas a decrease in signal intensity with increasing temperature was observed for MARV VP35 (Figure 4C). On the basis of these results, a 4:1 M ratio between GFP 9M6 and GFP 10M2/11M4-tagged VP35 oligomerization domains, 60 min incubation time and 23°C incubation temperature were chosen to conduct all subsequent experiments.

Defective assembly of EBOV and MARV VP35 coiled coil correlates with loss of GFP FC

Next, we wanted to assess the suitability of the assay to study oligomerization-related VP35 gain- or loss-of-function arising from mutagenesis. To this aim, we co-expressed and purified the GFP 10M2/11M4-tagged EBOV VP3S L90A/L93A/L107A triple and MARV VP3S L90A/L104A double mutants (Figure S5A), which were previously found to be oligomerization-defective and reported to impair VP35 suppression of the host interferon response and virus replication, respectively (Reid et al., 2005; Möller et al., 2005). In agreement with previous results obtained for non GFP-tagged constructs bearing these mutations (Zinzula et al., 2019), biophysical characterization of GFP 10M2/11M4 EBOV VP3S L90A/L93A/L107A and GFP 10M2/11M4 MARV VP3S L90A/L104A oligomerization domains supported full disruption of the coiled-coil folding. In fact, SEC-MALS showed that both EBOV and MARV GFP-tagged VP35 mutants tend to multimerize into oligomeric forms of high MW (Figure S5B), suggesting that such multimers are the result of an aberrant assembly that does not rely on coiled-coil knobs-into-holes packing. In addition, Nano-DSF analysis of GFP 10M2/11M4-tagged EBOV VP3S L90A/L93A/L107A and MARV VP3S L90A/L104A mutants showed lower Tm values with respect to WT and two inflection points (EBOV, ~49°C and ~64°C versus ~77°C; MARV, 40 and ~60°C versus ~76°C), indicating that both these mutants are inherently less stable (Figures S5E and S5F). Furthermore, the in vivo FC screening agreed with biophysical experiments, as colonies of triple transformant E. coli cells expressing all three components of the split-GFP tripartite system displayed low fluorescence close to background level (Figure 4D). Also, in the in vitro assay, both co-expressed and purified GFP 10M2/11M4 EBOV VP3S L90A/L93A/L107A and GFP 10M2/11M4 MARV VP3S L90A/L104A oligomers failed to reconstitute a full-length GFP when incubated with the GFP 9M6 reporter, as shown by
Validation of tripartite split-GFP FC system for screening antiviral candidates perturbing EBOV and MARV VP35 coiled coil assembly

Finding a small-molecule compound is able to entirely disrupt the \( \alpha \)-helix interactions within a coiled-coil appears an arduous challenge. In fact, the hydrophobic interactions tightening the knobs-into-holes packing system, as well as the electrostatic ones clamping the \( \alpha \)-helices outer shell through inter-and intra-chain salt bridges, are highly stable and thermodynamically favored (Lupas and Bassler, 2017). Nonetheless, small molecule inhibitors of respiratory syncytial virus (Roymans et al., 2010), human immunodeficiency virus (McGee et al., 2017) and Middle East respiratory syndrome coronavirus (Kandeel et al., 2020) fusion proteins are all representative examples of the fact that even subtle perturbations of the coiled-coil helical properties and conformation arising from the binding of a ligand to its accessible pockets may have dramatic consequences on the domain function. With this in mind, we sought to evaluate how the local destabilization of the coiled-coil register – be it the result of a disruptive mutation or the interaction with a ligand – would affect the conformation at the distal portions of the oligomerization domain where the two GFP-tags are attached. To this aim, we first performed explorative molecular dynamics (MD) simulations on the solved crystallographic structures of the EBOV and MARV VP35 trimeric coiled-coils and on the modeled structure of the EBOV tetrameric one, comparing the WT constructs to their oligomerization-defective mutant counterparts. As shown by the analysis of MD trajectories (Figures S6 and S7), Leu to Ala substitutions at a and d positions of the coiled-coil hydrophobic core introduce irregularities into the knob-into-holes pairing system, leading the coiled-coil superhelix to partially unwind and the \( \alpha \)-helices to slightly depart from each other (Figures S8–S10). This rationalizes the failure of VP35 oligomers to keep their \( \alpha \)-helices in proper conformation, which is essential for GFP 10M2 and 11M4 tags to get in contact with the GFP 9M6 reporter and undergo FC. We then benchmarked the FC assay by using for pilot screening a set of small molecules (\( N = 8 \)) from an in-house library of chemical compounds (Figure S11A), and found that the two compounds Myricetin (Figure 5A) and 4,5,6,7-Tetramobenzotriazole (TBBT) (Figure 5B) inhibited the VP35 oligomerization-mediated split-GFP FC (Figures S12A and S12B). Myricetin prevented the coiled-coil of EBOV and MARV VP35 oligomerization domains from assuming their correct conformation, showing IC\(_{50}\) values of 64.4 and 25.5 \( \mu \)M, respectively (Figures 5C and 5E). Similarly, the inhibitory effect exerted by TBBT resulted in IC\(_{50}\) values of 32.5 and 9.8 \( \mu \)M, respectively (Figures 5D and 5F). Calculation of the assay \( Z' \)-factor yielded a value of 0.71, which is indicative of assay robustness (Zhang et al., 1999). Moreover, both Myricetin and TBBT neither displayed intrinsic fluorescence, nor diminished that of a full-length eGFP (Figure S11B). Instead, Nano-DSF showed that both compounds specifically interact with EBOV and MARV VP35 oligomerization domains. In fact, when tested on the VP35 constructs devoid of the GFP 10M2 and 11M4 tags at concentrations corresponding to the split-GFP FC IC\(_{50}\) value or higher in excess (e.g. 100 \( \mu \)M), Myricetin and TBBT were both able to induce a shift in the Tm of the native (non GFP-tagged) EBOV and MARV VP35 oligomerization domains (Figure S12C). In particular, the Tm value of both EBOV and MARV VP35 oligomerization domains was higher in the presence of Myricetin (EBOV: 70.8, 73.2 and 73.5\(^\circ\)C for 0, 64.5 and 100 \( \mu \)M Myricetin, respectively; MARV: 70.0, 74.3 and 75.4\(^\circ\)C for 0, 25.5 and 100 \( \mu \)M Myricetin, respectively) (Figures 2E, 2F, S13A, and S13B). Of interest, Myricetin was previously shown to intercalate into the hydrophobic core of the coiled-coil SNARE complex (Yang et al., 2010), and to increase the Tm value of rhodopsin, thereby enhancing its thermal stability upon rigidification of its \( \alpha \)-helical bundle (Ortega et al., 2019). Also, Myricetin was reported to interact with EBOV VP35 RBD/IID, while inhibiting its dsRNA binding and IFN antagonism functions (Daino et al., 2018; Corona et al., 2022). Within this picture, from the results here shown it is tempting to speculate that Myricetin may act on VP35 by concomitantly targeting two different domains, thereby having a synergistic inhibitory effect on its functionalities. Conversely, both EBOV and MARV VP35 oligomerization domains displayed lower Tm values in the presence of TBBT (EBOV: 70.8, 67.4 and 66.9\(^\circ\)C for 0, 32.5 and 100 \( \mu \)M TBBT, respectively; MARV: 70.0, 64.0 and 59.8\(^\circ\)C for 0, 10 and 100 \( \mu \)M TBBT, respectively) (Figures 2E, 2F, S13C, and S13D). Noteworthy, TBBT and its derivatives were previously reported to inhibit the activity of hepatitis C virus, West Nile virus and Japanese encephalitis virus NTPase/helicases (Borowski et al., 2003; Bretner et al., 2005). Of interest, such enzymatic function has been ascribed to the C-terminal amphipathic region downstream of the VP35 coiled coil (Shu et al., 2019), tempting to speculate that binding of TBBT to the VP35 decreases in the fluorescence signal by approximately 98 and 97%, respectively, as compared to WT (Figure 4E). Taken together, these results demonstrate that fluorescence of the complemented tripartite split-GFP depends on the nature of the side chains that interact in the EBOV and MARV VP35 coiled-coil, and that disruption or destabilization of the VP35 quaternary structure directly correlates with loss or reduction of GFP fluorescence (Figure S4B).
oligomerization domain may also affect its NTPase/helicase activity and suggesting further studies on this direction.

Prediction of Myricetin and TBBT mode of binding on EBOV and MARV VP35 coiled-coil assembly-mediated oligomerization

To gain insight on the mode of action of the two ligands, we performed blind molecular docking experiments. For the trimeric and tetrameric structures of the EBOV VP35 oligomerization domain, the best binding poses showed that Myricetin and TBBT insert into a pocket created by the intertwining of the α-helical

Figure 5. Validation of Split-GFP FC system for antiviral screening of compounds interfering with EBOV and MARV VP35 coiled-coil assembly-mediated oligomerization

(A) Chemical structure of Myricetin and (B) TBBT.

(C) Dose-response inhibition curve by Myricetin on the FC of split-GFP driven by oligomerization of GFP 10M2/11M4 EBOV VP35, IC_{50} = 24.4 μM.

(D) Dose-response inhibition curve by TBBT on the FC of split-GFP FC driven by oligomerization of GFP 10M2/11M4 EBOV VP35, IC_{50} = 32.5 μM.

(E) Dose-response inhibition curve by Myricetin on the FC of split-GFP driven by oligomerization of GFP 10M2/11M4 MARV VP35, IC_{50} = 9.8 μM.

(F) Dose-response inhibition curve by TBBT on the FC of split-GFP FC driven by oligomerization of GFP 10M2/11M4 MARV VP35, IC_{50} = 17.4 μM.
chains across the second and the fourth heptad repeats of the coiled-coil canonical register, a region that overlaps with the residues at d positions Leu93 and Leu107 that are essential for proper knobs-into-holes pairing (Figures 6A, 7A, and S14). Similarly, for the trimeric structure of the MARV VP35 oligomerization domain, the best binding poses showed that both Myricetin and TBBT insert into a pocket that overlaps (or is adjacent to) the fifth (non-canonical) heptad repeat of the coiled-coil canonical register, whose position corresponds to that of Leu104, also critical for coiled-coil proper assembly (Figures 6B and 7B). Overall, results from molecular docking suggest that the inhibitory effect of Myricetin and TBBT toward FC of the split-GFP recapitulates, at least in part, the one resulting from the introduction of oligomerization-defective mutations such as L90A/L93A/L107A in EBOV VP35 and L90A/L104A in MARV VP35. By interposing between the coiled-coil helices, both compounds likely determine conformational modifications upstream and downstream of their point of insertion that somehow make the GFP 10M2 and 11M4 tags unable to properly interact with the GFP 9M6 detector. It may be tempting to speculate that Myricetin because of the more extensive hydrogen bond interactions established with residues at the pocket surface, could act by clamping and rigidifying the coiled coil, which would explain the enhancement in thermal stability observed in both EBOV and MARV VP35 oligomerization domains in the presence of the compound. Conversely, because of its smaller dimension and consequently lower steric hindrance, TBBT could act by prying in a lock pick-like fashion. Future investigations are however needed to support such interpretations.

In summary, this study demonstrates that EBOV and MARV VP35 oligomerization domains are amenable targets for the development of drugs that, by altering VP35 coiled-coil conformation, will inhibit the functions for which an oligomeric VP35 is crucial, such as filovirus genome replication and innate immune evasion. The VP35 tripartite split-GFP FC system represents therefore a new promising resource in the ‘antiviral research toolbox’ for the identification of novel drugs that, given the high conservation degree of the VP35 oligomerization domain across all filoviruses, may be also capable of pan-filoviral activity.

Limitations to the study
The tripartite split-GFP FC system is suitable to assess the impact of mutagenesis-induced defects in the VP35 oligomer formation, and also to screen for small molecules that may affect the conformational stability of the VP35 coiled-coil, among which Myricetin and TBBT have been proven to be exemplary starting models. However, further studies are needed to evaluate their binding kinetics and inhibitory effect in the context of a full-length VP35. In fact, one limitation to this study lies in the fact that it provides insights into the oligomerization of VP35 in a domain-specific perspective, with no experiments conducted on the full-length protein in a mammalian cytoplasmic environment. Although this element could mistakenly lead to perceive the study as of limited impact, it indeed represents its point of strength. In fact, the tripartite split-GFP FC allows us to dissect out the coiled-coil mediated VP35 oligomerization from the other properties of a multifunctional and modular protein as VP35 is, whose multimerization via other domains in the full-length construct would disturb readout interpretation and inevitably mask the real potential of antiviral candidates targeting the coiled-coil mediated VP35 oligomerization, an indispensable process for the filoviral life cycle. Nevertheless, although the system can in principle be adapted to mammalian cell cultures and fluorescence imaging, it is intended to be used as an agile biochemical tool for initial screening and then to be coupled to orthogonal, well established cell-based methods such as mini-genomes or other reporter assays, as well as to experiments that use live viruses at the highest biocontainment level.

STAR+METHODS
Detailed methods are provided in the online version of this paper and include the following:

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Figure 6. Molecular docking model and interaction map for Myricetin binding to EBOV and MARV VP35 oligomerization domains

(A) Cartoon representation (zoomed in the inset) of putative binding mode of Myricetin to the coiled-coil of the homo-trimeric EBOV VP35 oligomerization domain (upper left panel) and two-dimensional (2D) diagram of predicted compound-residue interactions within the binding pocket (upper right panel).

(B) Cartoon representation (zoomed in the inset) of putative binding mode of Myricetin to the coiled-coil of the homo-trimeric MARV VP35 oligomerization domain (upper left panel) and 2D diagram of predicted compound-residue interactions within the binding pocket (upper right panel). Red, white and blue regions on the VP35 isosurface represent areas of negative, neutral, and positive electrostatic surface potential, respectively (scale of $-50$ to $+50$ kT $e^{-1}$); H-bonds are drawn as blue dashed lines with distances between atoms indicated in angstrom (Å); hydrophobic contacts are depicted as arcs with spokes; amino acid sequences and coiled-coil heptad registers are shown below each model (lower panels), with critical Leucines at $a$ and $d$ positions highlighted in black, and residues interacting with Myricetin indicated by lime-green lines.
Figure 7. Molecular docking model and interaction map for TBBT binding to EBOV and MARV VP35 oligomerization domains.

(A) Cartoon representation (zoomed in the inset) of putative binding mode of TBBT to the coiled-coil of the homo-trimeric EBOV VP35 oligomerization domain (upper left panel) and 2D diagram of predicted compound-residue interactions within the binding pocket (upper right panel).

(B) Cartoon representation (zoomed in the inset) of putative binding mode of TBBT to the coiled-coil of the homo-trimeric MARV VP35 oligomerization domain (upper left panel) and 2D diagram of predicted compound-residue interactions within the binding pocket (upper right panel). Red, white and blue regions on the VP35 isosurface represent areas of negative, neutral, and positive electrostatic surface potential, respectively (scale of $-50$ to $+50 \text{ kT e}^{-1}$); H-bonds are drawn as blue dashed lines with distances between atoms indicated in angstrom ($\text{Å}$); hydrophobic contacts are depicted as arcs with spokes; amino acid sequences and coiled-coil heptad registers are shown below each model (lower panels), with critical Leucines at $a$ and $d$ positions highlighted in black, and residues interacting with TBBT indicated by lime-green lines.
Far-UV CD
Nano-DSF
In vivo whole cell split-GFP FC screening
In vitro split-GFP FC assay
Homology modeling
MD simulations
Molecular docking
Molecular graphics and data statistical analysis

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105354.

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AUTHOR CONTRIBUTIONS
L.Z. conceived the study. L.Z. and I.N. designed and performed molecular cloning. L.Z., A.M.M., and C.S. performed protein expression and purification and carried out all biochemical and biophysical experiments. L.Z and M.O. performed MD simulations and molecular docking. L.Z., I.N., M.O., and A.B. performed data analysis and interpretation. W.B. supervised experimental design and data interpretation. L.Z. drafted the manuscript. All authors contributed to experimental design, data analyses and manuscript review.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduction of research.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| E. coli 5-alpha | New England BioLabs | Cat #C2987H |
| E. coli BL21 (DE3) | New England BioLabs | Cat #C25271 |
| **Chemicals, peptides, and recombinant proteins** | | |
| LB Broth (Miller) | Carl-Roth | Cat #X968.4 |
| Agar-Agar, bacteriological | Carl-Roth | Cat #2266.1 |
| di-Potassium hydrogen phosphate trihydrate | Carl-Roth | Cat #6878.1 |
| Potassium dihydrogen phosphate | Carl-Roth | Cat #3904.1 |
| Sodium dihydrogen phosphate dihydrate | Carl-Roth | Cat #T879.1 |
| di-Sodium hydrogen phosphate dihydrate | Carl-Roth | Cat #4984.3 |
| Sodium chloride | Sigma-Aldrich | Cat #53014 |
| Ampicillin sodium Salt | Sigma-Aldrich | Cat #A9518 |
| Kanamycin sulfate | SERVA | Cat #26899.03 |
| Spectinomycin sulfate | Enzo Life Sciences | Cat #BML-A281-0010 |
| IPTG | Carl-Roth | Cat #CN08.4 |
| Glycerol | Sigma-Aldrich | Cat #G5516 |
| Imidazole | Merck | Cat #1047160250 |
| Lysozyme from chicken egg white powder | Sigma-Aldrich | Cat #62971 |
| cOmplete EDTA-free Protease Inhibitor Cocktail | Roche | Cat #11873580001 |
| DNase I from bovine pancreas | Roche | Cat #10104159001 |
| TCEP | TRC Canada | Cat #T012500 |
| Trizma®-hydrochloride | Sigma-Aldrich | Cat #93363 |
| Magnesium chloride | Sigma-Aldrich | Cat #M8266 |
| Cerivastatin sodium salt hydrated ≥ 98% | Sigma-Aldrich | Cat #5ML0005 |
| Cymarin ≥ 98% | Supelco | Cat #91801 |
| Ivermectin ≥ 98% | Sigma-Aldrich | Cat #I8898 |
| Myricetin ≥ 96% | Sigma-Aldrich | Cat #M6770 |
| Prasugrel ≥ 98% | Sigma-Aldrich | Cat #5ML0331 |
| Suramin Sodium salt ≥ 98% | Sigma-Aldrich | Cat #52671 |
| TBBT ≥ 98% | Sigma-Aldrich | Cat #T0826 |
| TMC353121 ≥ 98% | MedChemExpress | Cat #HY-11097 |
| eGFP | This work | N/A |
| BSA | ThermoFisher | Cat #AM2616 |
| NuPAGE™ 4–12% Bis-Tris protein gels | ThermoFisher | Cat #NP0232 |
| NuPAGE™ LDS Sample Buffer | ThermoFisher | Cat #NP0007 |
| NuPAGE™ Antioxidant | ThermoFisher | Cat #NP0005 |
| NuPAGE™ Sample Reducing Agent | ThermoFisher | Cat #NP0004 |
| NuPAGE™ MES SDS Running Buffer | ThermoFisher | Cat #NP0002 |
| PageRuler™ Prestained Protein Ladder | ThermoFisher | Cat #26616 |
| **Recombinant DNA** | | |
| pET-21b (+) DNA-Novagen | Merck Millipore | Cat #69741 |
| pETDuet-1 DNA-Novagen | Merck Millipore | Cat #71146 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pRSFDuet-1 DNA-Novagen | Merck Millipore | Cat #71341 |
| pCDFDuet-1 DNA-Novagen | Merck Millipore | Cat #71340 |
| pET-21b-EBOV-VP35(75–185) | This work | N/A |
| pET-21b-MARV-VP35(58–174) | This work | N/A |
| pETDuet-GFP-9M6 | This work | N/A |
| pRSFDuet-GFP-10M2-EBOV-VP35(75–185) WT | This work | N/A |
| pCDFDuet-GFP-11M4-EBOV-VP35(75–185) WT | This work | N/A |
| pRSFDuet-GFP-10M2-EBOV-VP35(75–185) L90A/L93A/L107A | This work | N/A |
| pCDFDuet-GFP-11M4-EBOV-VP35(75–185) L90A/L93A/L107A | This work | N/A |
| pRSFDuet-GFP-10M2-MARV-VP35(85–174) WT | This work | N/A |
| pCDFDuet-GFP-11M4-MARV-VP35(58–174) WT | This work | N/A |
| pRSFDuet-GFP-10M2-MARV-VP35(58–174) L90A/L104A | This work | N/A |
| pCDFDuet-GFP-11M4-MARV-VP35(58–174) L90A/L104A | This work | N/A |

Deposited data

- Crystal Structure of Split GFP complexed with engineered sfCherry with an insertion of GFP fragment
  - Nguyen et al., 2013
  - PDB: 4KF5

- Crystal structure of the oligomerization domain of Vp35 from Ebola virus
  - Zinzula et al., 2019
  - PDB: 6GBO

- Crystal structure of the Marburg Virus VP35 Oligomerization Domain P4222
  - Bruhn et al., 2017
  - PDB: STOI

Software and algorithms

- PSIPRED
  - Buchan and Jones, 2019
  - http://bioinf.cs.ucl.ac.uk/psipred/

- ASTRA 6
  - Wyatt Technology
  - https://www.wyatt.com/products/software/astra.html

- Spectra Manager
  - Jasco
  - https://www.jasco.de/Spectroscopy/Software

- PR.ThermControl
  - NanoTemper Technologies
  - https://nanotempertech.com/prometheus-pr-thermcontrol-software/

- QuestGraph™ IC₅₀ calculator
  - AAT Bioquest
  - https://www.aatbio.com/tools/ic50-calculator

- Clustal Omega
  - Madeira et al., 2019
  - https://www.ebi.ac.uk/Tools/msa/clustalo/

- MODELLER 10.1
  - Webb and Sali, 2016
  - https://salilab.org/modeller/

- MolProbity
  - Williams et al., 2018
  - http://molprobity.biochem.duke.edu

- SOCKET
  - Walshaw and Woolfson, 2001
  - http://coiledcoils.chm.bris.ac.uk/socket/server.html

- QwikMD
  - Ribeiro et al., 2016
  - http://www.ks.uiuc.edu/Research/qwikmd/

- NAMD
  - Phillips et al., 2020
  - https://www.ks.uiuc.edu/Research/namd/

- SOCKET2
  - Kumar and Woolfson, 2021
  - http://coiledcoils.chm.bris.ac.uk/socket2/home.html
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Luca Zinzula (zinzula@biochem.mpg.de).

Materials availability
The plasmids and cell lines generated in this study are available from the lead contact and will be subjected to a material transfer agreement.

Data and code availability
The datasets generated in this study are available from the lead contact on reasonable request. This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell cultures
Chemically-competent E. coli NEB® 5-alpha and BL21-DE3 cells were purchased from New England Biolabs, transformed with the corresponding plasmids, cultured in Luria-Bertani (LB) and Terrific broth (TB) medium, respectively, and supplemented with 15% Glycerol for long-term storage at –80°C.

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| PubChem             | Kim et al., 2021 | https://pubchem.ncbi.nlm.nih.gov |
| Open Babel          | O’Boyle et al., 2011 | http://openbabel.org/wiki/Main_Page |
| AutoDock Vina       | Trott and Olson, 2010 | https://vina.scripps.edu |
| CB-Dock             | Liu et al., 2020 | http://clab.labshare.cn/cb-dock/php/index.php |
| LIGPLOT             | Wallace et al., 1995 | https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/ |
| Chimera             | Pettersen et al., 2004 | https://www.cgl.ucsf.edu/chimera/ |
| PyMOL               | Schrödinger | http://www.pymol.org/ |
| Tm-align            | Zhang and Skolnick, 2005 | https://zhanglab.ccmbr.med.umich.edu/TM-align/ |
| MatchMaker          | Meng et al., 2006 | https://www.cgl.ucsf.edu/chimera/docs/ContributedSoftware/matchmaker/matchmaker.html |
| PDB2PQR             | Dolinsky et al., 2007 | https://www.poissonboltzmann.org |
| APBS                | Baker et al., 2001 | https://www.poissonboltzmann.org |
| ChemDraw            | PerkinElmer | https://perkinelmerinformatics.com/products/research/chemdraw |
| MolView             | MolView | https://molview.org |
| Prism 9             | GraphPad | https://www.graphpad.com |

Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HisTrap FF crude 1mL | GE Healthcare | Cat #11-004-58 |
| Superose 12 10/300 GL | GE Healthcare | Cat #17-5173-01 |
| Superdex 200 10/300 GL | GE Healthcare | Cat #17-5175-01 |
| Precision cuvette highly pure quartz glass Suprasi™ 110-QS | Helima | Cat #110-1-40 |
| Prometheus NT.48 Series nanoDSF Grade NanoTemper Standard Capillaries | NanoTemper Technologies | Cat #PRC002 |
| 96-well Black Flat Bottom Polystyrene NBS Microplate | Corning | Cat #3991 |
| 384-well Low Flange Black Flat Bottom Polystyrene NBS Microplate | Corning | Cat #3375 |
**METHOD DETAILS**

**Molecular cloning, protein expression and purification**

Boundaries of EBOV and MARV VP35 oligomerization domains were designed after secondary structure prediction using the PSIPRED (Buchan and Jones, 2019) server. Codon-optimized cDNAs encoding for WT and mutant L90A/L93A/L107A EBOV (Zaire ebolavirus, Yambuku-Mayinga, GenBank: NC_002549.1, residues 75–185) and WT and mutant L90A/L104A MARV (Marburg marburgvirus, Elgon-Musoke, GenBank: NC_001608.3, residues 58–174) VP35 oligomerization domains, native or N-terminally fused to GFP beta-strands 10 and 11 and for GFP beta-strands 1–9 (PDB: 4KFS, chain C and A, respectively) (Nguyen et al., 2013) were all obtained by custom synthesis (Eurofins Genomics), cloned into modified pET-21b, pRSFDuet, pCDFDuet and pETDuet plasmid vectors (Novagen), respectively, and propagated ot maintained for long-term storage upon transformation of chemically competent *E. coli* NEB® 5-alpha (New England Biolabs) cells. Recombinant His6-tagged proteins were expressed or co-expressed in *E. coli* BL21-DE3 (New England Biolabs) cells grown in TB medium supplemented with 50mgmL⁻¹ Kanamycin and/or 50mgmL⁻¹ Spectinomycin and/or 100mgmL⁻¹ Ampicillin at 37°C to an OD 600 nm of 0.8, upon induction with 0.65–0.75 mM isopropyl-b-D-1-thiogalactopyranoside (IPTG) overnight (ON) at 25°C. Harvested cells were lysed in buffer A (50 mM sodium phosphate, pH 8.0; 500 mM NaCl; 5% (v/v) glycerol; 20 mM imidazole) supplemented with 1 mg mL⁻¹ Lysozyme (Sigma-Aldrich), cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and DNase I (Roche), then sonicated and centrifuged at 30,000g at 4°C for 30 min. The supernatant was loaded onto a 1 mL HisTrap FF crude (GE Healthcare) column equilibrated in buffer A and connected to an AKTA™ pure FPLC system (GE Healthcare). Column washing and protein elution were performed in buffer A containing 40 mM and 900 mM imidazole, respectively. SEC was performed on a Superose 12 10/300 GL (GE Healthcare) column in buffer B (25 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.2 mM MgCl₂) connected to an AKTA™ pure FPLC system (GE Healthcare). Protein purity and homogeneity were assessed by 4–12% NuPAGE SDS-PAGE (ThermoFisher). Protein identities and integrity were confirmed by WB and LC/MS. Purified full-length recombinant eGFP was kindly provided by the Protein Production Core Facility of the Max-Planck Institute of Biochemistry ( Martinsried, Germany).

**SEC-MALS**

SEC-purified main peak fractions of co-expressed protein samples (0.5 mg) were loaded on a Superdex 200 10/300 GL (GE Healthcare) column connected to a 1100 series HPLC system (Agilent Technologies), with its variable UV absorbance detector set to 280 nm and coupled in line to a mini DAWN TREOS MALS detector followed by an Optilab rEX refractive-index detector (Wyatt Technology, 690 nm laser) (Some et al., 2019). Runs were performed at 23°C and 0.75mLmin⁻¹ flow rate in buffer B supplemented with 1 mM tris(2-carboxyethyl)-phosphine (TRC Canada). Absolute molecular mass was calculated by using bovine serum albumin (BSA) (ThermoFisher) as calibration standard and the Astra 6 software (Wyatt Technology) with the dn/dc value set to 0.185mLg⁻¹.

**Far-UV CD**

CD measurements were recorded on a J-715 spectropolarimeter equipped with a PDF 3505 Peltier thermostat (Jasco) using a high-precision quartz cuvette of 1-mm path length (Hellma). Protein samples were analyzed at 0.1mgmL⁻¹ in buffer B. Spectra were recorded averages of four accumulations in the 195–250 nm wavelength range, at 23°C, 50nmmin⁻¹ scanning speed and 1 s response time. Mean molar per residue ellipticity (MRE) was calculated according to MRE as equal to 3300 mD A(lcn) where m is the molecular mass in Dalton, A is the Absorbance, l is the pathlength in cm, n the residue number, and c the protein concentration in mg mL⁻¹ (Greenfield, 2006). Data were processed by using the Spectra Manager software (Jasco), the CONTINLL deconvolution method and the SMPS6 reference set to estimate protein α-helical content.

**Nano-DSF**

For thermal unfolding profiles, protein samples were diluted to 3.0–4.5mgmL⁻¹ in buffer B, loaded on standard capillaries and subjected to a linear 20–95°C thermal gradient at 0.5°C min⁻¹ rate using a Prometheus NT.48 Nano-DSF instrument (NanoTemper Technologies). The temperature-dependent shift in intrinsic fluorescence at emission wavelengths of 330 and 350 nm was measured, and inflection points of fluorescence transition corresponding to the Tm values were determined as the first derivative maxima of the fluorescence intensities ratio at the measured wavelengths (F330/F350). Protein-ligand interaction studies were performed as described by Vivoli and colleagues (Vivoli et al., 2014). Myricetin (≥96%,...
Sigma-Aldrich) and TBBT (≥ 98%, Sigma-Aldrich) were tested at fixed concentration in excess (100 μM C₀) or equal to the IC₅₀ value obtained in the in vitro split-GFP FC assay, and against a fixed amount of protein. After incubation of 60 min at RT, sample quadruplicates or triplicates were tested and Tₘ values were averaged from at least three independent measurements, then processed using the PR.ThermControl software (NanoTemper Technologies).

**In vivo whole cell split-GFP FC screening**

Transformed *E. coli* BL21 (DE3) cells, co-expressing WT and mutant EBOV or MARV GFP 10M2/11M4 VP35 oligomerization domains and GFP 9M6, or singly expressing each component of the tripartite system, the full length eGFP or untagged VP35 oligomerization domains as positive and negative controls, respectively, were grown to saturation at 37 °C ON in LB medium supplemented with the appropriate combination of antibiotics. After induction with 1 mM IPTG, an aliquot (50–100 μL) of each ON culture was seeded on LB agar plates containing the same antibiotic combination and 0.75 mM IPTG, and incubated at 37 °C for 24 h. Induced colonies on plates were screened for fluorescence intensity and imaged on an iBright FL1500 imaging system (ThermoFisher Scientific) set to green LED (470–550 nm) transillumination and 0.11 s exposure time.

**In vitro split-GFP FC assay**

Reactions were set up as quadruplicates or triplicates in 100 μL or 50 μL volume in buffer B on 96 or 384-well low-flange, black flat bottom microplates (Corning), respectively. WT and mutant EBOV and MARV GFP 10M2, 11M4 or GFP 10M2/11M4 VP35 oligomerization domains at 2.3 μM final concentration (C₀) were tested singly or mixed with an excess of GFP 9M6 (C₁ of 10 μM for initial read-out screening), ranging from 36.8 μM to 0.575 μM Cᵢ for determination of the optimal 9M6:10M2:11M4 ratio and equal to 9.2 μM Cᵢ in all subsequent experiments, respectively. Reactions were incubated on a THERMOstar orbital shaker (BMG LabTech) set to 300 rpm, for 15–240 min at 4 °C, 23 °C and 37 °C for determination of the optimal incubation time and temperature, and for 60 min at 23 °C in all subsequent experiments, respectively. The fluorescence signal was measured as the average of total counts (subtracting the background fluorescence of buffer B as blank) using a Synergy HTX Multi-Mode microplate reader (Bio-Tek) equipped with filters for 485 and 528 nm excitation and emission wavelengths, respectively. Full-length eGFP and GFP 9M6 complemented with GFP 10M2/11M4 of WT EBOV and MARV VP35 oligomerization domains, GFP 9M6, GFP 10M2- and GFP11M4-fused, as well as native WT and mutant EBOV and MARV VP35 oligomerization domains were all used as negative controls. The Z² factor was calculated as described by Zhang et al. (1999), as Z² = 1–3(SDₛ² + SDₚ²)/(Mₛ + Mₚ), where SDₛ is the standard deviation of the positive control signal, SDₚ is the standard deviation of the negative control background, Mₛ is the mean of the positive control signal, and Mₚ is the mean of the negative control background (Zhang et al., 1999). For antiviral screening validation, a pilot set of compounds including Cerivastatin, Ivermectin, Prasugrel, Suramin, TBBT (≥ 98%, Sigma-Aldrich), Cynarin (≥ 98%, Supelco), Myricetin (≥ 96%, Sigma-Aldrich) and TMC353121 (≥ 98%, MedChemExpress) was tested by incubating each of those alone or in the presence of full-length eGFP, or pre-incubating each of those with GFP 10M2/11M4 VP35, for 60 min at 23 °C. Initial test was performed at 100 μM C₀ under the determined optimal assay parameters, then positive hits Myricetin and TBBT were tested at increasing concentrations ranging from 0.16 μM to 2500 μM C₀. After further incubation with GFP 9M6, fluorescence counts were measured and plotted as percentages of positive control. A non-linear regression fitting model was applied to the raw experimental data and IC₅₀ values were determined with the QuestGraph™ (AAT Bioquest) IC₅₀ calculator server (https://www.aatbio.com/tools/ic50-calculator).

**Homology modeling**

Homology models of putative tetrameric EBOV VP35 (WT and L90A/L93A/L107A mutant) and MARV VP35 (WT and L90A/L104A mutant) oligomerization domains were generated by using the RESTV VP35 ortholog as template (PDB: 6GBQ) (Zinzula et al., 2019). Pairwise sequence alignment was generated by using Clustal Omega (Madeira et al., 2019), visually inspected and fed into MODELLER 10.1 (Webb and Sali, 2016) for model building using the model-multichain.py routine. For each construct the final structure was selected from an output of thirty models based on the MODELLER objective function. Ramachandran plot and evaluation of side chains clashes evaluation were calculated using the MolProbity (Williams et al., 2018) web server.
MD simulations
Coiled-coil boundaries in the solved homo-trimeric EBOV (PDB: 6GBO, residues 83–117) (Zinzula et al., 2019) and MARV (PDB: 5TOI, residues 78–109) (Bruhn et al., 2017) VP35 oligomerization domains, as well as in their homology-modelled homo-tetrameric versions, were defined using the SOCKET (Walshaw and Woolfson, 2001) software. For the WT and as well as in silico generated L90A/L93A/L107A and L90A/L104A mutants, coiled-coils were prepared for the MD runs using QwikMD (Ribeiro et al., 2016) software. Systems were solvated in a 0.15 M NaCl water box, then energy-minimized, equilibrated and subjected to all-atoms production simulations of 200 ns at 37 °C using the NAMD (Phillips et al., 2020) software. Trajectories were analyzed by the QwikMD (Ribeiro et al., 2016) software. Evaluation of fluctuating residues between WT and mutant VP35 coiled-coils was performed considering as threshold the cut off value in the difference of root-mean-squared fluctuation (ΔRMSF) of ±0.5 Å, as previously described (Meher and Wang, 2015). Post-simulation analysis of coiled-coil conformation and register, knobs-into-holes packing and inter-helix and knobs angles was performed using SOCKET2 server (Kumar and Woolfson, 2021).

Molecular docking
Myricetin (CID: 5281672) and TBBT (CID: 1694) atomic coordinates were retrieved from PubChem (Kim et al., 2021) as SDF files and converted to PDB format by using Open Babel web server (O’Boyle et al., 2011). Blind docking experiments of both ligands on the solved trimeric EBOV (PDB: 6GBO) (Zinzula et al., 2019) and MARV (PDB: 5TOI) (Bruhn et al., 2017) VP35 oligomerization domains, as well as on their respective tetrameric homology models, were performed by using the AutoDock Vina algorithm (Trott and Olson, 2010) on the CB-Dock (Liu et al., 2020) web server. Vina score, cavity size, docking center and size of predicted cavity parameters were all set to default values. Best poses were selected and analyzed for bi-dimensional visualization of residue-ligand interactions, using the LIGPLOT software (Wallace et al., 1995).

Molecular graphics and data statistical analysis
Structural alignments, superpositions and depictions of proteins and ligands were produced with the Chimera v.1.13.1 (Pettersen et al., 2004) and PyMOL v.2.4 software (http://www.pymol.org/). Structural comparison between WT and mutant EBOV and MARV VP35 coiled-coils was performed by using TM-align (Zhang and Skolnick, 2005) and the MatchMaker (Meng et al., 2006) tools as implemented in the Chimera software (Pettersen et al., 2004). Poisson–Boltzmann electrostatic surface potential was calculated with the PARSE force field in PDB2PQR (Dolinsky et al., 2007) and APBS (Baker et al., 2001) as implemented in the PyMOL software (http://www.pymol.org/). Chemical structures of ligands were produced with the ChemDraw software (PerkinElmer) and the MolView server (https://molview.org).

QUANTIFICATION AND STATISTICAL ANALYSIS
Plotting and analysis of experimental data was done by use of the Prism 9 software (GraphPad). Data points and standard errors refer to averages (mean, SD) of replicates from at least three independent experiments.