**Alfalfa dwarf cytorhabdovirus P protein is a local and systemic RNA silencing
suppressor which inhibits programmed RISC activity and prevents transitive
amplification of RNA silencing**

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25 ABSTRACT

26 Plants employ RNA silencing as an innate defense mechanism against viruses. As a
27 counter-defense, plant viruses have evolved to express RNA silencing suppressor proteins
28 (RSS), which target one or more steps of the silencing pathway. In this study, we show
29 that the phosphoprotein (P) encoded by the negative-sense RNA virus alfalfa dwarf virus
30 (ADV), a species of the genus *Cytorhabdovirus*, family *Rhabdoviridae*, is a suppressor of
31 RNA silencing. ADV P has a relatively weak local RSS activity, and does not prevent
32 siRNA accumulation. On the other hand, ADV P strongly suppresses systemic RNA
33 silencing, but does not interfere with the short-distance spread of silencing, which is
34 consistent with its lack of inhibition of siRNA accumulation. The mechanism of suppression
35 appears to involve ADV P binding to RNA-induced silencing complex proteins AGO1 and
36 AGO4 as shown in protein–protein interaction assays when ectopically expressed. *In*
37 *planta*, we demonstrate that ADV P likely functions by inhibiting miRNA-guided AGO1
38 cleavage and prevents transitive amplification by repressing the production of secondary
39 siRNAs. As recently described for lettuce necrotic yellows cytorhabdovirus P, but in
40 contrast to other viral RSS known to disrupt AGO activity, ADV P sequence does not
41 contain any recognizable GW/WG or F-box motifs, which suggests that cytorhabdovirus P
42 proteins may use alternative motifs to bind to AGO proteins.

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44 Keywords:

45 Alfalfa dwarf cytorhabdovirus; Phosphoprotein; RNA silencing suppression; Protein-protein
46 interactions; Argonaute proteins; transitivity

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1. Introduction

Viruses are intracellular obligate parasites that exploit host cellular and molecular mechanisms to propagate in host cells causing many important plant diseases (Gergerich and Dolja, 2006). RNA silencing is a sequence-specific RNA-mediated degradation mechanism, a function of which is antiviral defense (Ding and Voinnet, 2007). RNA silencing is triggered by viral double-stranded RNA (dsRNA) (Voinnet, 2001) that, in the case of single-stranded RNA (ssRNA) viruses, can be derived from highly structured regions in the RNA genome or may be generated during replication (Molnar et al., 2005). DsRNA that accumulate during virus infection are processed into short dsRNAs of 21 to 24 nucleotide (nt), called small interfering RNAs (siRNAs), by members of the RNase III-like enzymes family, named DICER-like (DCL) proteins (Deleris et al., 2006). The siRNAs are loaded onto Argonaute (AGO) proteins, the effectors of the RNA-Induced Silencing Complex (RISC), to initiate slicing or translational repression of target RNAs (Ding and Voinnet, 2007; Carbonell and Carrington, 2015). A family of ten AGO proteins was identified in *Arabidopsis thaliana* (Vaucheret, 2008), whereas nine AGO homologs were identified in the *Nicotiana benthamiana* transcriptome (Nakasugi et al., 2013). Antiviral activity is associated mainly with AGO1, the core component of RISC in the siRNAs-mediated silencing pathways (Baumberger and Baulcombe, 2005; Mallory and Vaucheret, 2010; Qu et al. 2008), AGO4 also has slicer activity (Qi et al., 2006), and an antiviral function of this protein was proposed during cucumber mosaic virus (CMV) infection in *N. benthamiana* (Ye et al., 2009) and other RNA viruses (Carbonell and Carrington, 2015). AGO2, AGO5 and AGO7 have also been shown to have a role in antiviral defense (Brosseau and Moffett, 2015; Garcia-Ruiz et al., 2015). Once RNA silencing is initiated in a plant cell, primary siRNAs can be amplified through a process referred to as transitive silencing, which involves generation of secondary siRNAs extending from the primary targeted regions of a transcript, which is thought to be responsible for the systemic or long-

76 distance spread of the RNA silencing signal to target RNAs in distant tissues (Dunoyer et
77 al., 2010; Himber et al., 2003; Moissiard, et al., 2007). Silencing spread throughout a plant
78 requires RDR6-mediated amplification of ssRNA into dsRNA, which is subsequently
79 processed by DCL2 into transitive, secondary siRNAs (Himber et al., 2003, Mlowtshwa et
80 al., 2008). RDR6 activity is assisted by protein co-factors such as Suppressor of gene
81 silencing 3 (SGS3) (Beclin et al., 2002), silencing defective 3 (SDE3) (Dalmay et al., 2001;
82 Himber et al., 2003; Garcia et al., 2012) and SDE5 (Hernandez-Pinzon et al., 2007).

83 To counteract RNA silencing defenses, viruses encode RNA silencing suppressor
84 (RSS) proteins (Ding and Voinnet, 2007). Plant viruses have evolved many different RSS,
85 more than 70 to date have been identified (Csorba et al., 2015). Viral RSS have no
86 obvious sequence similarity, are multifunctional and may target one or more steps in the
87 silencing pathway, such as viral RNA recognition, dicing, RISC assembly, or RNA
88 targeting and amplification, as reviewed by Burgyan and Havelda (2011), Csorba et al.,
89 (2015) and Incarbone and Dunoyer (2013). A central feature of the majority of known plant
90 viral RSS is their ability to bind to and sequester long and/or short dsRNAs, in order to
91 inhibit cleavage by DCL or RISC assembly, respectively (Csorba et al. 2007; Duan et al.,
92 2012; Lakatos et al. 2006; Landeo-Ríos et al., 2016; Mérai et al. 2006; Perez-Canamas
93 and Hernandez, 2015; Schnettler et al., 2010). The best-characterized RSS that employs
94 this strategy is the tombusvirus P19 protein, which binds to both siRNAs and miRNAs
95 (Lakatos et al. 2006). However, other RSS function by interacting with host proteins that
96 are either important components of the silencing machinery or proposed regulators of the
97 silencing pathway (Kong et al., 2014). This includes RSS that interact with AGO1 to block
98 siRNA loading, inhibit slicing activity, or inhibit translational repression (Azevedo et al.,
99 2010; Chiu et al., 2010; Csorba et al., 2010; Feng et al., 2013; Giner et al., 2010; Karran
100 and Sanfacon, 2014; Kumar et al., 2015; Mann et al., 2016a; Zhang et al., 2006). In
101 addition, tobacco rattle virus 16K and CMV 2b proteins interact with AGO4 to block RISC

102 assembly and both *in vitro* slicing activity and RNA-directed DNA methylation, respectively
103 (Fernández-Calvino et al., 2016; Hamera et al., 2012). Other RSS have been shown to
104 function by interacting with siRNA biogenesis machinery such as dsRNA-binding protein 4
105 (DRB4) leading to inhibition of dsRNA processing by DCL4 (Haas et al. 2008) or by
106 interacting with SGS3 and RDR6 (Okano et al. 2014).

107 Rhabdoviruses are enveloped negative-sense, ssRNA viruses, which infect diverse
108 animal and plant hosts (Jackson et al., 2005; Mann and Dietzgen, 2014). Like other plant
109 viruses, plant rhabdoviruses are expected to encode protein(s) with RSS activity, but data
110 on their identification and possible mode of action are limited. Among viruses of the genus
111 *Nucleorhabdovirus*, sonchus yellow net virus (SYNV) P protein was shown to have local
112 RSS activity but was not further characterized (Jackson et al., 2005), while rice yellow
113 stunt virus (RYSV) P6 was identified as a systemic RSS and shown to prevent RNA
114 silencing amplification by interacting with RDR6 (Guo et al., 2013). The RSS of only one
115 cytorhabdovirus, lettuce necrotic yellows virus (LNYV), has been investigated thus far.
116 LNYV P protein was shown to act as a weak local RSS that did not inhibit siRNA
117 accumulation but temporarily delayed systemic RNA silencing in *N. benthamiana* (Mann et
118 al., 2015). In addition, it was recently shown that LNYV P binds AGO1, AGO2, AGO4,
119 RDR6 and SGS3 proteins. Targeting multiple components of the RNA silencing pathway
120 LNYV P inhibited miRNA-guided AGO1 cleavage and translational repression, and RNA
121 silencing amplification (Mann et al., 2016a). Given the importance of plant virus RSS
122 function in the infection cycle and host interactions, we identified and examined the RSS of
123 alfalfa dwarf virus (ADV), a virus that combines properties of both cytoplasmic and nuclear
124 plant rhabdoviruses; ADV groups phylogenetically with cytorhabdoviruses, but its P protein
125 accumulates in the cell nucleus (Bejerman et al., 2015). We also explored mechanisms of
126 action of ADV RSS through study of targeted host components. The ADV genome consists
127 of 14,491 nucleotides and encodes six proteins in the order 3'-N (nucleoprotein) – P

128 (phosphoprotein) – P3 (movement protein) –M (matrix protein) – G (glycoprotein) – P6
129 (protein of unknown function) – L (polymerase) - 5' (Bejerman et al., 2015; Mann et al.,
130 2016b).

131 In this study, we examined the suppressor activity of the putative RSS ADV P and
132 P6. We present evidence that ADV P acts as a weak local RSS, which does not inhibit
133 siRNA accumulation, but acts as a strong systemic RSS. On the other hand, ADV P6
134 protein did neither suppress local nor systemic RNA silencing unlike nucleorhabdovirus P6
135 in RYSV. We also show that ADV P binds AGO1 and AGO 4 proteins, and inhibits miRNA-
136 guided AGO1 cleavage and translational repression. Moreover, we show that ADV P
137 prevents transitive amplification of RNA silencing by repressing the production of
138 secondary siRNAs.

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140

141 **2. Material and methods**

142 *2.1. Plasmid construction*

143 Gateway entry clones in pDONR221 for ADV N (used as negative control), P and
144 P6 ORFs (Bejerman et al., 2015) (Life Technologies, California, USA) were recombined
145 into plant expression destination vector pSITE-Flag-C1 (Chakrabarty et al., 2007) to yield
146 N-terminal flag epitope-tagged proteins, following instructions described in the Gateway
147 LR Clonase II Enzyme Mix kit (Life Technologies). For **bimolecular fluorescence**
148 **complementation (BiFC)** protein–protein interaction assays, Gateway entry clones in
149 pDONR221 for ADV P (Bejerman et al., 2015), AGO1b, AGO4a and RDR6 (kindly
150 provided by Prof Peter Waterhouse, Queensland University of Technology; Nakasugi et
151 al., 2013) and SGS3 were recombined into plant expression destination vectors pSITE-
152 BiFC- nEYFP-C1 and pSITE-BiFC-cEYFP-C1 to allow expression of these proteins as C-
153 terminal fusions to the amino- or carboxy-terminal halves of yellow fluorescent protein
154 (YFP), using Gateway LR Clonase II Enzyme Mix kit instructions (Life Technologies). For

co-localization studies ADV P was recombined into GFP-pSITE-C1, and AGO1b, AGO4a, RDR6 and SGS3 into RFP-pSITE-C1 (Chakrabarty et al., 2007). All p-SITE plant expressions vectors were kindly provided by A/Prof Michael Goodin, University of Kentucky, USA (See Table S1 for a list of plasmids used).

Recombinant plasmids were individually transformed into *Agrobacterium tumefaciens* LBA 4404.

2.2. Agroinfiltration of *Nicotiana benthamiana*

A. tumefaciens LBA 4404 cultures were diluted in infiltration buffer (10mM MES, 10mM MgCl₂) to a final OD₆₀₀ of 0.4-0.5 prior to agroinfiltration into the abaxial side of 4–5 week old GFP-expressing 16c *N. benthamiana* leaves (Ruiz et al., 1998) or wild type *N. benthamiana* as previously described (Lindbo, 2007). BiFC assays were carried out using transgenic *N. benthamiana* plants with cyan fluorescent nuclei (CFP-Histone 2B) (Martin et al., 2009). *Agrobacteria* harboring plasmids encoding ER-targeted GFP (mGFP5-ER; pBIN-mGFP5-ER) was used as reporter for both local and systemic RNA silencing assays, 171.1 GFP sensor (pUQC10193) and 171.2 GFP sensor (Mann et al., 2016a) for miRNA-AGO1 cleavage assays, sense GFP (pUQC214-GFP) and double-stranded hairpin GFP (dsGF; pUQC-251) for transitivity assays, and TBSV P19 (pUQC-10035) was used as positive RSS control in plants (Table S1). Local and systemic silencing assays both using mGFP5-ER (Table S1) were independently carried out at least three times. For local silencing assays, GFP expression was recorded at 4 days postinfiltration (dpi), while for systemic silencing assays, GFP expression was recorded at 20 dpi and leaves were counted as systemically silenced if complete or partial development of red autofluorescence was observed in upper non-infiltrated leaves. Leaves were counted as not systemically silenced (i.e., silencing was suppressed) if the green GFP fluorescence in upper non-infiltrated leaves was unchanged and clearly detectable. For transitivity

181 experiments, agrobacteria carrying dsGF plasmid were diluted to OD₆₀₀ of 0.08 prior to
182 mixing in equal volumes with both agrobacteria carrying sense GFP (OD₆₀₀ 0.8) and viral
183 protein (OD₆₀₀ 0.8) expression constructs.

184

185 2.3. Protein analysis

186 Soluble protein extracted from agroinfiltrated leaf tissue (20mg) was resuspended in
187 sample buffer (Laemmli, 1970) and separated on an SDS-12% or 7% (for AGO1-Myc
188 protein experiments) polyacrylamide gel. Separated proteins were transferred to PVDF
189 membrane (Millipore, Massachusetts, USA) and detected as previously described (Mann
190 et al., 2015). Protein sizes were estimated using the Page Ruler pre-stained protein ladder
191 (Thermo Fisher Scientific, Massachusetts, USA).

192

193 2.4. RNA analysis

194 Total RNA was extracted from agroinfiltrated leaf tissue (1 g) using Tri Reagent
195 (Sigma-Aldrich). For detection of *GFP* siRNAs and transitivity experiments total RNA was
196 separated into high and low molecular weight (HMW/LMW) RNA as previously described
197 (Mitter and Dietzgen, 2012). Total and HMW or LMW RNAs were transferred from
198 denaturing 1.0% agarose or 17% polyacrylamide gels, respectively, to a BrightStar-Plus
199 nylon membrane and were detected with BrightStar BioDetect Kit (Life Technologies) as
200 previously described (Mann et al., 2015) or with the Chemiluminescent Nucleic Acid
201 Detection Module Kit (Thermo Fisher Scientific). *GFP* mRNA from local and systemic
202 silencing experiments was detected using 8 µg of total RNA. *GFP* sensor (171.1 and
203 171.2) and *GFP* mRNA from transitivity experiments were detected using 8 µg of total and
204 HMW RNA, respectively. *AGO1*-Myc mRNA was detected using 14 µg of total RNA. *GFP*
205 siRNAs were detected using 3–5 µg of LMW RNA. Unless otherwise stated, biotin-labeled
206 *GFP* (720 nt; full-length), *AGO1* (992 nt; corresponding to nt position 330–1322) and “*GF*”

(10–384 nt of *GFP*) and “*P*” (413–717 nt of *GFP*) DNA probes were generated as described by Mann et al., 2016a. Total RNA sizes were estimated using RiboRuler High Range RNA ladder (ThermoFisher Scientific) in conjunction with a linear regression analysis and siRNAs size by loading mixtures of *GFP*-specific short DNA oligonucleotides.

2.5. *GFP* fluorescence and confocal microscopy

GFP fluorescence in *N. benthamiana* leaves was observed using a long wavelength Black Ray model B 100AP UV lamp (UVP, California, USA) at 365 nm and images were captured using Canon EOS 450D camera equipped with a *GFP* filter. All microscopy was carried out on a Zeiss LSM-700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 2 and 3 dpi using at least three leaves for each of three independent biological replicates (nine leaves total) for BiFC experiments. Images were processed using Zen 2012 Lite software.

3. Results

Initial results showed that ADV N, P3, M and G proteins were not able to suppress RNA silencing (data not shown). Therefore, we examined whether ADV phosphoprotein (P) and protein 6 (P6) had RNA silencing suppressor (RSS) activity as shown for LNYV P (Mann et al., 2015) and RYSV P6 (Guo et al., 2013). Since various suppressor proteins target different steps in the silencing pathways, the stage at which P and/or P6 may act to suppress RNA silencing was also investigated.

3.1 *ADV P* but not *P6* acts as a local silencing suppressor in *N. benthamiana* 16c

An *Agrobacterium* leaf patch assay using at least 20 green fluorescent protein (*GFP*)-transgenic *Nicotiana benthamiana* line 16c plants, which constitutively express mGFP5-ER (Ruiz et al., 1998), was carried out to investigate whether ADV P and P6 act

233 as local RSS (see Table S1 for a list of plasmids used). Co-infiltrations of mGFP5-ER and
234 empty expression vector or tomato bushy stunt virus (TBSV) P19 RSS were used as
235 negative and positive controls, respectively. At 4 dpi, infiltrated leaves were examined
236 using a hand-held UV lamp and photographed. Stronger green fluorescence was observed
237 in the leaf patches expressing ADV P than in the patches expressing either ADV P6 or
238 empty vector, although to a lesser extent than in those expressing TBSV P19 (Fig. 1A);
239 however at 7 dpi no more silencing suppression was observed in those leaf patches
240 expressing ADV P (Fig. S1). Transient expression of flag-tagged ADV P and P6 proteins
241 was confirmed by western blot analysis at 4 dpi (Fig. S2). The visual results observed at 4
242 dpi were confirmed by western blot analysis of the GFP protein (Fig. 1D). Accumulation
243 levels of GFP protein were significantly higher in leaf patches co-infiltrated with the *GFP*
244 construct and TBSV P19 or ADV P constructs, than those expressing empty vector or ADV
245 P6, which showed reduced GFP levels due to silencing of *GFP* mRNA. We observed a
246 8.3-fold lower GFP protein accumulation in the leaf patches expressing ADV P when
247 compared to TBSV P19 (Fig. 1D). Consistent with the protein data, RNA blot analysis
248 showed that the steady-state level of *GFP* mRNA in the patches expressing ADV P was
249 higher than in those expressing either empty vector or ADV P6, but lower than in the
250 patches expressing TBSV P19 (Fig. 1B). These observations suggest that ADV P acts as
251 a RSS, but that its effect on local GFP silencing was considerably less efficient than that of
252 TBSV P19. TBSV P19 RSS activity led to the accumulation of at least 5-fold more *GFP*
253 RNA than the other constructs tested (Fig. 1B, note that 5 times less total RNA was loaded
254 for the p19 sample in the northern blots). This finding is in agreement with the GFP
255 fluorescence intensity observed in leaf patches and accumulation of GFP protein when
256 comparing ADV P to TBSV P19 (Fig. 1B). Taken together, these data provide evidence
257 that ADV P, but not ADV P6, functions as weak local RSS.

258

259 3.2 *ADV P* protein does not prevent *GFP* siRNA accumulation in *N. benthamiana* 16c

260 We next examined the generation of GFP-derived siRNAs to determine whether
261 *ADV P* affects their accumulation and to understand how this protein suppresses RNA
262 silencing in plants. By 4 dpi, RNA blots showed that silencing of *GFP* mRNA (Fig. 1B) in
263 tissue agroinfiltrated with GFP plus empty vector constructs or *ADV P6* correlated with a
264 strong accumulation of *GFP*-specific siRNAs (Fig. 1C). *GFP*-specific siRNA accumulation
265 was not significantly altered in *ADV P* co-expressing patches, compared with those
266 patches where the empty vector was co-expressed with mGFP5-ER (Fig. 1C). In contrast,
267 accumulation of *GFP* siRNAs was negligible in the presence of TBSV P19, which is known
268 to sequester siRNAs (Lakatos et al., 2006). These results indicate that *ADV P*, unlike
269 TBSV P19, does not appear to prevent the accumulation of siRNAs and thus likely targets
270 a step in the RNA silencing pathway downstream of primary siRNA biogenesis.

271
272 3.3 *ADV P* does not effect the short distance movement of the silencing signal in *N.*
273 *benthamiana* 16c

274 It has been reported that locally-induced RNA silencing can spread from initially
275 silenced cells up to 10-15 neighboring cells through the cell-to-cell movement of siRNAs,
276 which in leaves of *N. benthamiana* 16c line can be visualized under UV light as a narrow
277 red halo surrounding the infiltrated area (Himber et al., 2003). To examine the effect of
278 *ADV P* and *P6* on the short-distance spread of *GFP* silencing, the formation of this red
279 zone was monitored at 7 dpi in *N. benthamiana* 16c leaves infiltrated with mGFP5-ER plus
280 *ADV P*, *ADV P6*, TBSV P19 or empty vector (see Table S1 for a list of plasmids used).
281 Consistent with previous reports (Himber et al., 2003), the expression of TBSV P19
282 blocked the short-distance spread of the silencing signal, and thus, no red halo was
283 observed around patches infiltrated with TBSV P19 (Fig. S1). In contrast, a red zone was
284 consistently observed in those patches expressing *ADV P*, *ADV P6* or the empty vector
285 (Fig. S1). This data indicates that neither *ADV P* nor *ADV P6* interfered with the short-

286 distance spread of the silencing signal, which is consistent with its lack of inhibition of
287 siRNA accumulation.

288

289 3.4 ADV P but not P6 acts as a systemic silencing suppressor in *N. benthamiana* 16c

290 Once local RNA silencing is initiated in plants, the silencing signal can spread long
291 distance and induce systemic silencing of homologous sequences in upper leaves (Himber
292 et al., 2003), which in 16c plants is characterized by the vein proximal silencing of GFP in
293 newly emerging leaves. To determine whether ADV P or P6 interferes with this process,
294 GFP fluorescence was monitored at 20 dpi in upper leaves of *N. benthamiana* line 16c
295 plants infiltrated with mGFP5-ER plus either ADV P or ADV P6. We used empty
296 expression vector or TBSV P19 as negative and positive controls, respectively (see Table
297 S1 for a list of plasmids used). All 16c plants agroinfiltrated in three independent
298 experiments with mGFP5-ER and the empty vector (24/24) showed systemic GFP
299 silencing (i.e., red auto-fluorescence in upper non-infiltrated leaves) (Fig. 2A). Similarly, all
300 but two (22/24) 16c plants (92%) that were agroinfiltrated with mGFP5-ER and ADV P6
301 constructs showed systemic GFP silencing (Fig. 2A). In contrast, GFP silencing was
302 observed in only 12.5% (3/24) of 16c plants that were agroinfiltrated with mGFP5-ER and
303 ADV P constructs, while silencing was observed in just 4.2% (1/24) plants agroinfiltrated
304 with mGFP5-ER and TBSV P19 constructs (Fig. 2A). Northern blot analysis indicated that
305 in upper leaves of 16c plants that had been infiltrated 20 days earlier with GFP plus ADV P
306 or TBSV P19 constructs, systemic GFP silencing was suppressed and significant levels of
307 *GFP* mRNA had accumulated (Fig. 2B). The accumulation of *GFP* mRNA in upper leaves
308 of plants infiltrated with GFP plus ADV P6 constructs was similar to the upper leaves of
309 those plants co-infiltrated with the empty vector (Fig. 2B). Altogether, these data suggest
310 that ADV P protein is a strong suppressor of systemic RNA silencing similar to TBSV P19,
311 whereas ADV P6 protein does not appear to have systemic silencing suppressor activity.

312

313 3.5 ADV P interacts with NbAGO1 and NbAGO4 in planta

314 To shed further light on how ADV P mechanistically functions as an RSS, we
315 examined its potential interactions with components downstream of siRNA biogenesis. We
316 tested whether ADV P would co-localize and/or interact with RISC components AGO1 and
317 AGO4 when transiently co-expressed in *N. benthamiana* leaves. *N. benthamiana* genes
318 AGO1 and AGO4 (kindly provided by Dr. Peter Waterhouse, Queensland University of
319 Technology) (Nakasugi et al., 2013) were cloned into pSITE-RFP-C1, and individually
320 expressed as RFP fusions. At 2 dpi, confocal microscopy of agroinfiltrated *N. benthamiana*
321 leaves showed that RFP-AGO1 fusions accumulated uniformly on the cell periphery and in
322 the nucleus, while RFP-AGO4 was nuclear (Mann et al., 2016a). Next, we examined the
323 localization of these RFP fusions when co-expressed with GFP-ADV P, which localizes to
324 the nucleus (Bejerman et al., 2015). The partial cell periphery accumulation of RFP-AGO1
325 was no longer detectable when co-expressed with GFP-P, while the nuclear expression
326 remained. On the other hand, AGO4 signal in the nucleus appeared to be mostly excluded
327 from a large subnuclear region, where GFP-ADV P accumulated when RFP-AGO4 was
328 co-expressed with GFP-P (Fig. S3).

329 To determine if the observed re-localization effects by ADV P on AGO1 subcellular
330 distribution involved heterotypic protein–protein interactions, BiFC assays were done. At 2
331 dpi, ADV P showed clear interactions with AGO1 and AGO4 within the cell nucleus as
332 large and small aggregates, respectively (Fig. 3). As a control, ADV N was also tested but
333 failed to show any detectable interaction with AGO1 or AGO4 (data not shown). None of
334 the tested proteins interacted with GST negative control (Fig. 3). Collectively, these data
335 indicate that ADV P can interact with AGO1 and AGO4 proteins in the nucleus, but this will
336 need to be further validated with additional independent protein-protein interaction assays.

337

338 3.6 ADV P protein **likely** inhibits miRNA-mediated AGO1 cleavage and translational
339 repression

340 Targeting of AGO proteins by RSS has been shown to cause inhibition of slicer
341 activity (Azevedo et al., 2010; Fernández-Calvino et al., 2016; Giner et al., 2010; Kumar et
342 al., 2015; Mann et al., 2016a; Zhang et al., 2006). We hypothesized that ADV P may also
343 interact with AGO1 and/or AGO4 to disrupt slicer activity of assembled RISC complexes.
344 To test whether ADV P inhibits miRNA-loaded RISC complexes *in planta*, we co-
345 expressed GFP sensor constructs with or without a viral suppressor (Csorba et al., 2010;
346 Fernández-Calvino et al., 2016; Giner et al., 2010; Lakatos et al., 2006; Mann et al.,
347 2016a; Pantaleo et al., 2007; Parizotto et al., 2004). The GFP sensor construct,
348 pUQC10193 (referred to henceforth as “171.1”), transiently expresses a *GFP* gene that
349 contains a miR171 recognition site directly after the *GFP* open reading frame stop codon
350 (Fig. 4A) (Christie et al., 2011). Expression of the GFP sensor construct in *N. benthamiana*
351 leaves triggers silencing of *GFP* mRNA by the slicer activity of AGO1 loaded with
352 endogenous miR171 which can be observed by reduction of GFP fluorescence under UV
353 light (Giner et al., 2010; Lakatos et al., 2006; Mann et al., 2016a; Parizotto et al., 2004).
354 We also used a cleavage-resistant GFP sensor control construct, referred to as 171.2
355 (Mann et al., 2016a), which was obtained by mutating the 11th nucleotide in the miR171
356 recognition site of 171.1 as previously described (Parizotto et al., 2004). In a leaf patch
357 agroinfiltration assay at 4 dpi, GFP fluorescence and mRNA levels were low for 171.1 but
358 high for 171.2 when co-expressed with empty vector (Fig. 4B and C). This shows that the
359 GFP sensor 171.1 was functional and could be cleaved, while the mutated control 171.2
360 was cleavage-resistant. Elevated GFP fluorescence and mRNA levels were reproducibly
361 observed when either 171.1 or 171.2 were co-expressed with ADV P (Fig. 4B and C).
362 These results indicate that ADV P **likely** inhibits slicing activity of miR171-guided AGO1
363 RISC. We could not test if ADV P also inhibits siRNA-guided RISC due to lack of an

364 infectious clone of ADV. Interestingly, we noted that ADV P protein sequence did not
365 contain any GW/WG or F-box motifs (data not shown) which are typically present in viral
366 RSS that disrupt AGO activity (Csorba et al., 2015; Incarbone and Dunoyer, 2013).

367 The above results provide evidence that ADV P protein interacts with AGO1 protein
368 to likely inhibit slicer activity. To determine if ADV P protein interacts with AGO1 at the
369 protein or RNA level, we co-expressed Flag-tagged ADV P and Myc- tagged AGO1 *in*
370 *planta* and examined AGO1 mRNA and protein accumulation (Fig. 4D). At 4 dpi, northern
371 blot analysis of *N. benthamiana* leaves agroinfiltrated with AGO1-Myc showed a slightly
372 higher AGO1 mRNA accumulation in those patches where ADV P was co-expressed
373 compared with those patches where ADV N or empty vector were co-expressed (Fig 4D).
374 Protein blot analysis of the same samples showed that AGO1-Myc protein accumulation
375 was significantly increased when co-expressed with ADV P, compared to co-expression
376 with ADV N or empty vector (Fig. 4D). Taken together, our data provide evidence that ADV
377 P protein stabilizes AGO1 protein in RISC without significantly affecting steady state levels
378 of AGO1 mRNA.

379

380 3.7 ADV P protein inhibits amplification of RNA silencing

381 To determine if ADV P inhibits the amplification of RNA silencing, we carried out
382 GFP transitivity assays. In these assays, full-length sense *GFP* was co-expressed with a
383 “GF”-specific dsRNA hairpin construct corresponding to nucleotides 9–400 of the 720 nt
384 *GFP* coding sequence (Fig. 5A). Co-expression of these two vectors results in the
385 generation of “P”-specific transitive siRNAs corresponding to sequences downstream of
386 nucleotide 400 of the *GFP* transcript (Himber et al., 2003). Due to the known high potency
387 of GFP silencing induction by dsGF, we determined that diluting the solution of
388 agrobacteria carrying the dsGFconstruct by a factor of ten ($OD_{600} = 0.08$) allowed for
389 weaker RSS activities to be identified more reliably than in a standard assay (data not

shown). At 4 dpi, GFP fluorescence was maintained only when GFP plus dsGF was co-expressed with ADV P (weak fluorescence) or TBSV P19 (strong fluorescence) but not for the empty vector (Fig. 5B). Consistent with the GFP fluorescence data, northern blot analysis of agroinfiltrated leaf patches showed that ADV P expression on average led to a 2-fold higher *GFP* mRNA accumulation, whereas TBSV P19 expression led to a 15-20-fold higher *GFP* mRNA accumulation (Fig. 5C). The level of primary “GF” siRNA in ADV P expression patches appeared lower than that in the vector expression patches (Fig. 5C). However, ADV P-expressing tissues showed almost no accumulation of secondary “P” siRNAs, whereas TBSV P19-expressing tissues did not accumulate any detectable secondary siRNAs. Since ADV P does not appear to prevent the accumulation of siRNAs (Fig. 1C), the above data suggests that ADV P likely prevents transitive amplification of RNA silencing by interfering with the production of secondary siRNAs.

3.8 ADV P does not interact with RDR6 or SGS3 in planta

The ability of ADV P protein to likely prevent transitive amplification of RNA silencing by suppressing the production of secondary siRNAs suggests its interference with components of amplification of RNA silencing, which involves the concerted activity of RDR6 and SGS3 (Glick et al., 2008; Guo et al., 2013). Therefore, we tested whether ADV P would co-localize and/or interact with RDR6 and SGS3 when transiently co-expressed in *N. benthamiana* leaves. *N. benthamiana* genes RDR6 and SGS3 were individually expressed as fusions to the C-terminus of RFP. At 2 dpi, confocal microscopy of agroinfiltrated *N. benthamiana* leaves showed that RFP-RDR6 accumulated on the cell periphery and in the nucleus (Fig. S4A), while RFP-SGS3 formed small aggregates on the cell periphery (Mann et al., 2016a). No major change in intracellular localization was observed for RFP-RDR6 or RFP-SGS3 when co-expressed with GFP-ADV P (Fig. S4B). When BiFC assays were performed ADV P failed to show any detectable interaction with

416 RDR6 or SGS3 (Fig. S4C). Both plant proteins appeared to be correctly expressed,
417 because we observed a cytoplasmic interaction between *N. benthamiana* RDR6 and
418 SGS3 similar to that previously shown for *A. thaliana* counterparts (Kumakura et al., 2009).
419 Taken together, our results appear to indicate that ADV P may be targeting different host
420 factors other than RDR6 and SGS3, involved in secondary siRNA biogenesis, such as
421 DCL2, SDE3 or SDE5.

422

423 4. Discussion

424 In this study, using an *in vivo* co-agroinfiltration approach, we present evidence that
425 the phosphoprotein (P) encoded by the cytorhabdovirus ADV suppresses local RNA silencing
426 without affecting primary siRNA accumulation, suggesting effects on downstream steps of
427 the pathway. We further provide evidence that ADV P local RSS functions by arresting
428 RISC activity through binding to argonaute proteins and likely preventing RNA slicing. We
429 also show that ADV P suppresses systemic silencing by inhibiting the production of
430 secondary siRNAs. On the other hand, ADV P6 did not suppress either local or systemic
431 silencing.

432 In agroinfiltration leaf patch assays, ADV P local GFP silencing suppression activity
433 appeared visually weaker than that of TBSV P19, which was supported by mRNA
434 accumulation levels (Fig.1). ADV P weak local RSS activity was similar to that reported for
435 the P protein of the cytorhabdovirus LNYV (Mann et al., 2015), and several other plant
436 virus RSS including beet necrotic yellow vein virus p14, rice yellow mottle virus P1,
437 chinese wheat mosaic virus cysteine-rich protein (CRP), PVX P25 and potato virus M
438 12kDa CRP, all of which also exhibit weak RSS activity in leaf patch assays (Andika *et al.*,
439 2012; Fusaro et al., 2012; Senshu et al., 2009; Senshu et al., 2011; Sun et al., 2013).
440 Interestingly, like ADV P, LNYV P and PVX P25 have been shown to target multiple

441 components of the RNA silencing pathway by suppressing AGO1 and AGO4 activity and
442 systemic silencing (Chiu et al., 2010; Mann et al., 2016a; Voinnet et al., 2000).

443 Unlike TBSV P19 and most of the known silencing suppressors, which sequester
444 siRNAs (Burgyan and Havelda, 2011; Csorba et al., 2015), ADV P does not prevent GFP
445 siRNA accumulation (Fig. 1). This indicates that ADV P may exert its suppressor activity
446 downstream of primary siRNA biogenesis, similar to LNYV P (Mann et al., 2015).

447 Although ADV P is a weak local RSS (Fig. 1) and is not able to block the cell-to-cell
448 spread of the systemic silencing signal, consistent with its lack of sequestering siRNAs
449 (Fig. S1), this protein efficiently suppressed systemic silencing (Fig. 2), whereas LNYV P
450 RSS delayed but did not prevent systemic silencing (Mann et al., 2015). ADV P6 does not
451 noticeably suppress either local or systemic RNA silencing (Fig. 1, 2), unlike its counterpart
452 P6 of the nucleorhabdovirus RYSV, which was shown to suppress systemic RNA silencing
453 efficiently by inhibiting RDR6-mediated secondary siRNA production (Guo et al., 2013).
454 Apparently, RSS activity can differ even among analogous proteins encoded by viruses in
455 the same genus or family. This is also evidenced by P0 of different poleroviruses
456 (Kozłowska-Makulska et al., 2010), as well as the P25 of several potexviruses (Senshu et
457 al., 2009) that show different levels of RSS activity. The function of ADV P6 remains
458 unknown.

459 It was recently shown that at least the last 20 amino acids of LNYV P protein C-
460 terminal domain is required for both local RNA silencing suppression and interaction with
461 AGO1 and AGO4 (Mann et al., 2016a). Preliminary data suggest that C-terminal portion of
462 ADV P is also likely to be involved in local RSS activity. Future studies using ADV P
463 mutants may confirm this and will determine more generally if the C-terminal portions of
464 cytorhabdovirus P proteins may represent the active domains of RSS activity.

465 AGO slicer protein in the RISC degrades target RNAs by direct cleavage or
466 translational repression (Brodersen et al., 2008; Mallory and Vaucheret, 2010). RSS can

467 be grouped based on whether they inhibit either RISC assembly or preassembled RISC
468 complexes. ADV P does not appear to sequester siRNAs (Fig. 1), and therefore is unlikely
469 to prevent siRNA loading into RISC. Our experimental evidence suggests that ADV P likely
470 inhibits miRNA-guided AGO1 cleavage (Fig. 4) through direct protein–protein interaction
471 (Fig. 3). Co-infections with an infectious ADV clone system, once available, could be used
472 for siRNA-guided cleavage tests in the future. Nevertheless, it has been reported that other
473 viral RSS that inhibit assembled AGO1-RISC activity impaired with similar efficiency
474 siRNA or miRNA loaded AGO1-RISC (Schott et al., 2012), tempting us to speculate that
475 ADV P may also inhibit siRNA-loaded RISC complexes *in planta*, as reported for SPMMV
476 P1 (Giner et al., 2010).

477 It has been suggested that GW/WG motifs are essential for the RSS function of
478 TCV P38 and SPMMV P1, which disrupt miRNA-mediated AGO1 cleavage (Azevedo et
479 al., 2010; Giner et al., 2010). GW motifs are found in Argonaute binding proteins, such as
480 Tas3, NRPE1 and RNA Pol V, and AGO-GW interactions have been shown to be
481 important to initiate RISC assembly (El-Shami et al., 2007). However, *in silico* analysis of
482 ADV P did not detect any sequence resembling the GW/WG motifs. Other AGO- inhibiting
483 viral RSS, such as the P0 of poleroviruses and enamoviruses have been shown to encode
484 F- box motifs, which are thought to be involved in the degradation of AGO1 via autophagy
485 (Baumberger et al., 2007; Derrien et al., 2012; Fusaro et al., 2012). ADV P sequence does
486 not contain any identifiable F-box motifs. Furthermore, ADV P appears to enhance rather
487 than diminish AGO1 protein accumulation (Fig. 4), the opposite effect to that observed with
488 P0 (Baumberger et al., 2007; Fusaro et al., 2012). Similarly, no recognizable GW/WG or F-
489 box motifs were identified in LNYV P sequence (Mann et al., 2016a), suggesting that
490 cytorhabdovirus P proteins may represent a novel family of AGO-binding proteins. Further
491 studies using alanine-scanning mutagenesis could be used to determine the motifs linked
492 with AGO binding and its RSS activity.

493 RNA silencing in plants is amplified with the help of host proteins such as RDR6,
494 SGS3, SDE3, and others such as DCL2 and SDE5, in a process known as transitivity.
495 Transitivity results in the synthesis of secondary siRNAs and is associated with promoting
496 an effective RNA silencing response against viruses (Garcia et al., 2012; Hernandez-
497 Pinzon et al., 2007; Himber et al. 2003; Mlowtshwa et al., 2008; Moissiard et al., 2007;
498 Parent et al., 2015). In this study, ADV P showed a strong and reproducibly detectable
499 suppression activity of secondary siRNA synthesis (Fig. 5), whereas LNYV P showed a
500 comparatively weaker activity (Mann et al., 2016a). LNYV P, similar to the potexvirus
501 PIAMV P25, bound to both RDR6 and SGS3 (Mann et al., 2016a; Okano et al., 2014). The
502 majority of RSS known to suppress RNA silencing amplification appear to target either
503 RDR6 (Guo et al., 2013) or SGS3 (Glick et al., 2008; Rajamaki et al., 2014). However,
504 ADV P failed to show any detectable interaction with RDR6 or SGS3 in BiFC assays.
505 Okano et al. (2014) failed to detect an interaction between PIAMV P25 and RDR6 using
506 BiFC but this interaction was detected using co-IP assays. These authors suggested that
507 the reason that they did not detect the interaction using the BiFC assay might have been
508 due to the low expression level of RDR6. However, Mann et al., (2016a) using a similar
509 BiFC assay and the same RDR6 expression construct used here were able to detect an
510 interaction between RDR6 and LNYV P. Alternatively, ADV P could be targeting a different
511 host factor involved in secondary siRNA biogenesis, like DCL2, SDE3 or SDE5. Both
512 SDE3 and DCL2 are localized in the nucleus (Garcia et al., 2012; Xie et al., 2004), where
513 ADV P also accumulates (Bejerman et al., 2015).

514 AGO1-mediated cleavage would allow RDR6 recruitment to generate secondary
515 siRNAs (Voinnet, 2008). Therefore, it is tempting to speculate that the inhibition of AGO1
516 by ADV P may be associated with the suppression of transitive silencing, leading to
517 drastically reduced secondary siRNAs (Fig. 5). A similar scenario was proposed for the
518 poleroviral P0 that shown to interfere with AGO1 activity (Fusaro et al., 2012).

519 The strong inhibition of transitivity by RYSV P6 has been proposed to be connected
520 to its ability to strongly suppress systemic silencing (Guo et al., 2013). On the other hand,
521 LNYV P suppresses transitivity weakly, which might be linked with its capability to delay
522 but not prevent systemic silencing (Mann et al., 2016a). Therefore, it seems reasonable to
523 speculate that the strong suppression of transitivity by ADV P may be linked to its strong
524 suppression of systemic silencing (Fig. 2).

525 In summary, our data suggest that ADV P, like LNYV P (Mann et al., 2016a) targets
526 multiple steps in the RNA silencing pathway, but unlike LNYV P, ADV P exhibits a strong
527 systemic silencing activity. We hypothesize that the P protein of cytorhabdoviruses has
528 evolved to target multiple steps of the RNA silencing pathway, linked to their relative weak
529 local RSS activity. Hence, further detailed studies should be conducted to probe the
530 mechanism underlying the RSS activity of this protein, preferably using approaches that
531 resemble the natural context of the virus-plant pathosystem, like the use of a reverse
532 genetics system similar to the one recently developed for SYNIV (Wang et al., 2015).

533

534

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788

789 **Figure legends**

790

791 **Fig. 1.** Local GFP silencing suppression activity of ADV proteins. (A) *N. benthamiana* 16c
792 leaves expressing mGFP5-ER were co-agroinfiltrated with mGFP5-ER plus either ADV P
793 or P6. TBSV P19 or empty pSITE-Flag vector (V), were used as positive and negative
794 controls, respectively. Leaves were photographed for GFP fluorescence under long
795 wavelength UV light using a Canon EOS camera equipped with a GFP filter at 4 dpi.
796 Infiltrated leaf patches were analyzed for accumulation of *GFP* mRNA (B) and *GFP* siRNA
797 (C) using total and low molecular weight RNA, respectively. RNAs were detected using a
798 full-length *GFP*-specific biotin-labeled probe. Using soluble protein fractions, GFP (D) was
799 detected using an anti-GFP monoclonal antibody. Loading controls for *GFP* mRNA
800 (ribosomal RNA; rRNA), siRNA (5SRNA) and protein (RuBisCO) are shown below
801 respective blots. Note: all samples were loaded equally with the exception of TBSV P19 in
802 the *GFP* mRNA blot, which was diluted by a factor of 5 to reduce high signal intensity.
803 Relative accumulation levels in comparison to the empty vector reference (V) are shown
804 below respective lanes.

805

806 **Fig. 2.** Systemic GFP silencing suppression activity of ADV P and P6 proteins. (A) *N.*
807 *benthamiana* 16c plants expressing mGFP5-ER were co-agroinfiltrated with mGFP5-ER
808 plus either ADV P or P6. TBSV P19 or empty pSITE-Flag vector (V), were used as positive
809 and negative controls, respectively. Infiltrated 16c plants were visually examined and
810 scored for suppression of GFP silencing under UV light at 20 dpi. Systemic spread of
811 silencing to distal leaves of infiltrated plants was observed as vein proximal GFP silencing.
812 The number of plants that showed systemic silencing out of the total number of plants
813 tested is indicated in each panel. All images were taken adaxially under long wavelength
814 UV light using a Canon EOS camera equipped with a GFP filter. (B) Top fully expanded *N.*

815 *benthamiana* line 16c leaves were analyzed for accumulation of *GFP* mRNA using a full-
816 length mGFP5-ER-specific biotin-labeled probe. Ethidium bromide-stained rRNAs are
817 shown as loading controls. Relative accumulation levels in comparison to the empty vector
818 reference (V) are shown below respective lanes.

819

820 **Fig. 3.** Bimolecular fluorescence complementation (BiFC) of ADV P protein interactions
821 with RNA silencing proteins AGO1 and AGO4. Protein–protein interaction assays were
822 carried out in transgenic *N. benthamiana* expressing cyan fluorescent protein (CFP) fused
823 to the nuclear marker histone 2B (CFP-H2B). Shown are the localization of CFP-H2B
824 (nucleus, left column), interaction assay (BiFC, middle column) and a merge of both
825 channels (Overlay, right column). Proteins listed first in the pair of interactors were
826 expressed as fusions to the amino-terminal half of yellow fluorescent protein (YFP). Those
827 listed second were expressed as fusions to the carboxy-terminal half of YFP. However, all
828 pairwise combinations were tested but only a subset of those is shown here. Glutathione-
829 S-transferase (GST) was used as a non-binding control to validate interactions for all ADV
830 P interactors but only GST:P is shown here as a representative example. Live cell images
831 of agroinfiltrated leaves were taken at 2 dpi using a confocal microscope at 40x
832 magnification. The inset scale bar corresponds to 20 µm in all panels.

833

834 **Fig. 4.** miRNA-guided AGO1 slicer and AGO1 translational repression assays. *N.*
835 *benthamiana* leaves were co-agroinfiltrated with (A) a *GFP* sensor construct (171.1 –
836 sliceable or 171.2 – non sliceable) plus either empty vector or ADV P. (B) *GFP* sensor
837 infiltrated leaves were photographed for GFP fluorescence and (C) analyzed for
838 accumulation of *GFP* mRNA at 4 dpi. Leaf images were taken abaxially under long
839 wavelength UV light using a Canon EOS camera equipped with a GFP filter. **Relative**
840 **accumulation levels of *GFP* mRNA in comparison to the empty vector reference (V) are**

841 shown below respective lanes. (D) *N. benthamiana* leaves were co-agroinfiltrated with
 842 AGO1-Myc plus either Flag-tagged empty vector (denoted “V”), ADV N or ADV P. Infiltrated
 843 patches were analyzed at 4 dpi for AGO1 mRNA and protein accumulation, and for
 844 expression of ADV proteins. Using total RNA, *GFP* sensor and AGO1 mRNA were
 845 detected using a full-length GFP-specific or *N. benthamiana* AGO1-specific (corresponding
 846 to nt position 330–1322) biotin-labeled probe, respectively. Using soluble protein fractions,
 847 AGO1-Myc and ADV-Flag tagged proteins were detected using anti-Myc or anti-Flag
 848 monoclonal antibodies, respectively. Loading controls for mRNA (ribosomal RNA; rRNA),
 849 and protein (RuBisCo) are shown below respective blots. All samples were loaded equally
 850 for both RNA and protein blots, with the exception of ADV P for only the protein blot, of
 851 which 10 times less protein was loaded to reduce high signal intensity. Relative
 852 accumulation levels of AGO1 mRNA in comparison to the empty vector reference (V) are
 853 shown below respective lanes. RB/LB: right/left border; 35S: *cauliflower mosaic virus* 35S
 854 promoter; black bar in *GFP* gene indicates an intron; ocs: octopine synthase; BAR:
 855 glufosinate tolerance gene; nos: nos terminator.

856

857 **Fig. 5.** RNA silencing transitivity assay. (A) T-DNA maps of two-component *GFP* reporter
 858 used for transitivity assay, pUQC214-GFP and pUQC251-dsGF. (B) *N. benthamiana*
 859 leaves were co-agroinfiltrated with GFP (pUQC214-GFP) and double-stranded GF-specific
 860 hairpin constructs (pUQC251-dsGF) plus either ADV P, empty pSITE-Flag vector
 861 (“Vector”) or TBSV P19. Infiltrated leaves were photographed for GFP fluorescence at 4
 862 dpi. All images were taken adaxially under long wavelength UV light using a Canon EOS
 863 camera equipped with a GFP filter. (C) Infiltrated leaf patches involving co-agroinfiltration
 864 for mock (buffer), ADV P, TBSV P19 (“P19”) and empty vector (“V”) were analyzed for
 865 accumulation of *GFP* mRNA, primary plus secondary GF-specific siRNAs (“GF siRNAs”)
 866 and secondary transitive P specific siRNAs (“P siRNAs”). Total RNA was separated into

867 high (mRNA) and low (siRNA) molecular weight fractions, and RNA gel blot analysis was
868 done using a full-length GFP-specific or GF- and P-specific biotin probes, respectively.
869 Loading controls for *GFP* mRNA (ribosomalRNA; rRNA), siRNA (5SRNA), and relative
870 abundance of transcripts in comparison to the empty vector reference (V) are shown below
871 respective lanes. ADV P and empty vector were loaded equally, whereas for the *GFP*
872 mRNA blot only, the TBSV P19 positive control was diluted by a factor of five to reduce
873 high signal intensity (denoted “*”).

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5