Functional analysis of agrobacterium tumefaciens virulence protein VirD5

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Chapter 3

The VirD5 protein interferes with mitosis in yeast cells

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Abstract

The kinetochore is a large complex of multiple proteins that assembles at the centromeric region of the chromosome and serves to connect chromosomes to the spindle microtubules during mitosis. It thus plays crucial roles in faithful chromosome segregation. We found that the *Agrobacterium tumefaciens* virulence protein VirD5 is localized via its N-terminal part (NT) at the centromeres/kinetochores and targets the mitotic machinery to effect chromosome mis-segregation. Here, we report that VirD5NT interacts with the essential mitotic regulatory protein Ipl1/Aurora kinase and the outer kinetochore/microtubule-associated protein Dam1. Interaction with the Ipl1/Aurora kinase stimulates its kinase activity. Phosphorylation of substrates such as Dam1 is known to result in the detachment between kinetochore and spindle microtubule. This is necessary for error correction, but increased Ipl1/Aurora kinase activity could lead to spindle checkpoint activation, lagging chromosomes and in the end chromosome mis-segregation. That activation of the Ipl1/Aurora kinase underlies the toxicity of VirD5NT became apparent by artificial boosting of the activity of the specific counteracting phosphatase Glc7, which relieved the toxicity. Apparently, *A. tumefaciens* targets the conserved Aurora kinases to influence cell division and chromosome segregation in the initial phases of infection to advance tumorigenesis in plants.

Introduction

Faithful segregation of duplicated chromosomes over daughter cells during mitosis relies on the attachment of the kinetochore, a large complex of proteins that is made up of more than 60 proteins in yeast to the spindle microtubules (Cheeseman and Desai, 2008; Cho and Harrison, 2012). Structurally the kinetochore is divided into two distinct parts consisting of an inner kinetochore that directly binds to the centromeric DNA of the chromosome, and an outer kinetochore that attaches to the spindle microtubules (Hori *et al*., 2008; Yamagishi *et al*., 2014).

Chromosomes interact with the plus ends of spindle microtubules via their kinetochores and are segregated over daughter cells as the spindle microtubules are depolymerizing and shortening at the onset of anaphase (Kline-smith and Walczak, 2004). In budding yeast, a ten protein Dam1 complex and an Ndc80 complex play essential roles in maintaining the attachment between the kinetochore and the spindle microtubules (Hofmann *et al*., 1998). The Dam1 complex assembles into a 50-nm ring-like structure that embraces the microtubules (Miranda *et al*., 2005). The ring complex remains attached to the assembling tips of spindle microtubules and harness the energy from the depolymerization of microtubules against tension to facilitate chromosome movement during mitosis (Umbreit *et al*., 2014; Westermann *et al*., 2006). Although the Dam1 complex is exclusively present in fungi, a structurally similar Ska1 complex is present in human cells (Schmidt *et al*., 2012). The Dam1 complex binds to the kinetochores through a direct interaction with the Ndc80 complex, an essential outer kinetochore complex consisting of Ndc80, Nuf2, Spc24 and Spc25 (Lampert *et al*., 2013; Wigge and Kilmartin, 2001). The Ndc80 complex forms a rod-shaped heterotetramer that directly binds to the spindle microtubules by its N-terminal calponin homology (CH) domain and interacts with other kinetochore proteins by its C-terminal globular domain (Malvezzi *et al*., 2013).

Chromosomes with erroneous kinetochore-microtubule attachments stimulate the mitotic checkpoint to delay the onset of mitosis until all duplicated chromosomes are bipolarly attached (Sacristan and Kops, 2015). Correction of improper kinetochore-microtubule attachments is achieved by the Aurora B kinase, Ipl1 in budding yeast (Biggins *et al*., 1999; Saurin *et al*., 2011), a component of the Chromosomal Passenger Complex (CPC) further consisting of INCENP/Sli15, Survivin/Bir1 and Borealin/Nbl1 (Carmena *et al*., 2012). Ipl1/Aurora B kinase is thought to destabilize erroneous attachments via phosphorylation of key proteins including Ndc80/Hec1 and Dam1/Ska1 that are involved directly in the kinetochore-microtubule attachment (DeLuca, Lens, and DeLuca, 2011; Schmidt *et al*., 2012; Umbreit *et al*., 2014). Therefore, a reduction in the activity of Aurora B/Ipl1 leads to chromosome mis-segregation and aneuploidy. Overexpression of Aurora B/Ipl1, however, also leads to defective chromosome segregation as the repeated disruption of kinetochore-microtubule attachments not only activates the Spindle Assembly Checkpoint (SAC), but in the end generates lagging chromosomes and aneuploid cells (Etemad, Kuijt, and Kops, 2015; Muñoz-Barrera and Monje-Casas, 2014).

In our previous study, we found that the *A. tumefaciens* virulence protein VirD5 affects mitosis and causes chromosome mis-segregation in budding yeast. Here investigated further the molecular basis of the toxicity of VirD5 and found evidence that VirD5 directly interacts

with the conserved Ipl1/Aurora kinase and that by enhancing its kinase activities causes chromosome mis-segregation and aneuploidy.

Results

VirD5 directly binds to yeast centromeric DNA

The centromere is the chromosomal locus that is required for the assembly of the kinetochore and thus for the delivery of one copy of each chromosome into the daughter cells during mitosis. Centromeric DNA is extremely diverse among eukaryotes, ranging from the simple ~125bp centromere of *S. cerevisiae* to the highly repetitive α-satellite regions of vertebrates (Burrack and Berman, 2012; Carroll and Straight, 2006). It was shown that VirD5 contains several DNA binding motifs (Schrammeijer *et al*., 2000) and is localized at the centromeres/kinetochores in yeast nuclei (**Chapter 2**), and therefore, we wondered whether VirD5 could directly bind to the centromeric DNA of yeast. To test this, we carried out an Electrophoretic Mobility Shift Assay (EMSA) using the centromeric DNA from chromosome 3 (CEN3) and chromosome 16 (CEN16) as probes. Primers labeled by Cy5 at their 5'ends (**Table 1**) were used to amplify yeast CEN3 and CEN16 fragments. His-tagged VirD5 expressed and purified from *E.coli* was incubated either with labeled CEN3 or CEN16 *in vitro* for 30 minutes at room temperature, whereafter the DNA-protein mixtures were separated in a 2% agarose gel. As can be seen in **Figure 1A** and **B**, lanes 2, two shifted bands were clearly observed after VirD5 had been incubated with these centromeric DNA probes, whereas no extra band was visible in control experiments lacking VirD5 (**Figure 1A** and **B**, lanes 1). To confirm the specificity of the binding activity, non-labelled CEN3 or CEN16 were used as the competitive DNA. The presence of a 100-fold excess of competitor fragments completely eliminated the band shifts (**Figure 1A** and **B**, lanes 3). These data indicate that VirD5 can specifically bind to yeast centromeric DNA.

In budding yeast, the centromeric DNA consists of three regions named CDEI, CDEII and CDEIII, each of which is bound by distinct proteins (Hemmerich *et al*., 2000; Westermann, Drubin, and Barnes, 2007). CDEI, an 8 bp conserved DNA region (ATCACGTG), is recognized and bound by centromere binding factor 1 (CBF1) (Hemmerich *et al*., 2000); CDEII, a 78-86 bp core element consisting of AT-rich DNA, is specifically wrapped by nucleosomes with the histone 3 variant Cse4 (Camahort *et al*., 2007); CDEIII contains 25 bp that recruits the centromere binding factor 3 (CBF3) complex as a platform for inner kinetochore assembly (Lechner and Carbon, 1991). To determine which part of the centromeric DNA could be bound by VirD5, we digested the double Cy5 end-labelled CEN16 DNA with DraI (**Figure 1C**) to obtain two single-end labelled small fragments, which were subsequently separately incubated with His-tagged VirD5 *in vitro*. As shown in **Figure 1D**, VirD5 exclusively bound to the left region of CEN16 which contains CDEI and a part of CDEII.

VirD5 interacts with kinetochore-associated proteins

Kinetochores are large protein complexes that assemble only at centromeres and interact with spindle microtubules to establish bipolar attachment of paired sister chromatids during mitosis. Structurally, kinetochores consist of an inner kinetochore, which is bound directly to the centromere and an outer kinetochore, which mediates spindle microtubule attachment

(Burrack and Berman, 2012; Hemmerich *et al*., 2000). In budding yeast, each kinetochore is attached to only a single microtubule. Recently more than 60 yeast kinetochore proteins have been identified, many of which are conserved among eukaryotes (Biggins, 2013). To test whether VirD5 interacts with proteins which are associated with the kinetochore, we performed Bimolecular Fluorescent Complementation (BIFC) experiments (Kerppola, 2008). Candidate proteins (**Table 2**) from the inner and outer kinetochore were fused with the N-terminal part of YFP (VN173), and introduced into yeast cells together with VirD5 fused with the C-terminal part of YFP (VC173). The outer kinetochore/microtubule-associated protein Dam1 and the essential mitosis regulatory Ipl1/Aurora kinase displayed a very strong BIFC YFP signal with VirD5 (**Figure 2A**, a-c, g-i), while the fusion of Dam1or Ipl1 together with the unfused complementary part did not give a YFP signal (**Figure 2A**, d-f and j-l). No interactions with any of the other tested kinetochore proteins were observed (**Table 2**).

To confirm the interactions, an *in vitro* pull-down assay was carried out: Dam1 or Ipl1 protein fused to the GST tag was expressed in *E.coli* and bound to the Glutathione HiCap Matrix as the bait. The beads were incubated separately with His-tagged VirD5 purified from *E.coli*, for 2 hours at room temperature in binding buffer containing 0.1% Triton-100. After 3 times washing, the protein mixtures were separated on a SDS-PAGE gel. The presence of His-tagged VirD5 was detected by Western blot using anti-His antibody. As can be seen in **Figure 2C**, VirD5 physically interacted with GST-tagged Dam1 and GST-tagged Ipl1, but not with empty GST.

The interactions of VirD5 with Dam1 and Ipl1 requires Spt4

If interference with the functioning of the kinetochore is causing the toxicity of VirD5 its presence at the kinetochore would be imperative for toxicity. Deletion of the *spt4* gene altered the sublocation of VirD5 as the punctate foci in the nucleus disappeared (**Chapter 2**). We were therefore interested to find out whether the interactions of VirD5 with Dam1 and Ipl1 rely on Spt4 in the cell. To this end we introduced the BIFC vector 34VCn-VirD5 either with 35VNc-Dam1 or 35VNc-Ipl1 into the *spt4* deletion mutant to determine the BIFC signal. As shown in **Figure 2D**, no YFP signal was observed in *spt4* mutant cells expressing VirD5 together with either Dam1 or Ipl1, demonstrating that the interaction with Dam1 and Ipl1 is dependent on Spt4. The weak interactions of VirD5 with Ipl1 and Dam1 *in vitro* suggests that *E.coli* might have an Spt4-like elongation factor or charged amino acids could have provided an interaction site *in vitro* at high protein concentration.

VirD5 disturbs spindle microtubule elongation and chromosome segregation over the daughter cells

The Dam1 protein forms rings encircling the microtubules, that translate the force generated by depolymerization of the microtubules into movement of chromosomes attached via the kinetochore (Franck *et al*., 2007). In order to visualize the microtubule, we labelled tubulin with GFP. The spindle microtubule in wild type cells elongated into both mother and daughter cells during mitosis. In contrast, in the presence of VirD5, more than 80% of anaphase cells had short spindle microtubules that did not manage to enter the daughter cell (**Figure 3A**). To further confirm these spindle elongation defects caused by VirD5, we endogenously labeled the Dam1 protein with a 3xGFP tag at its C-terminus. Dividing cells

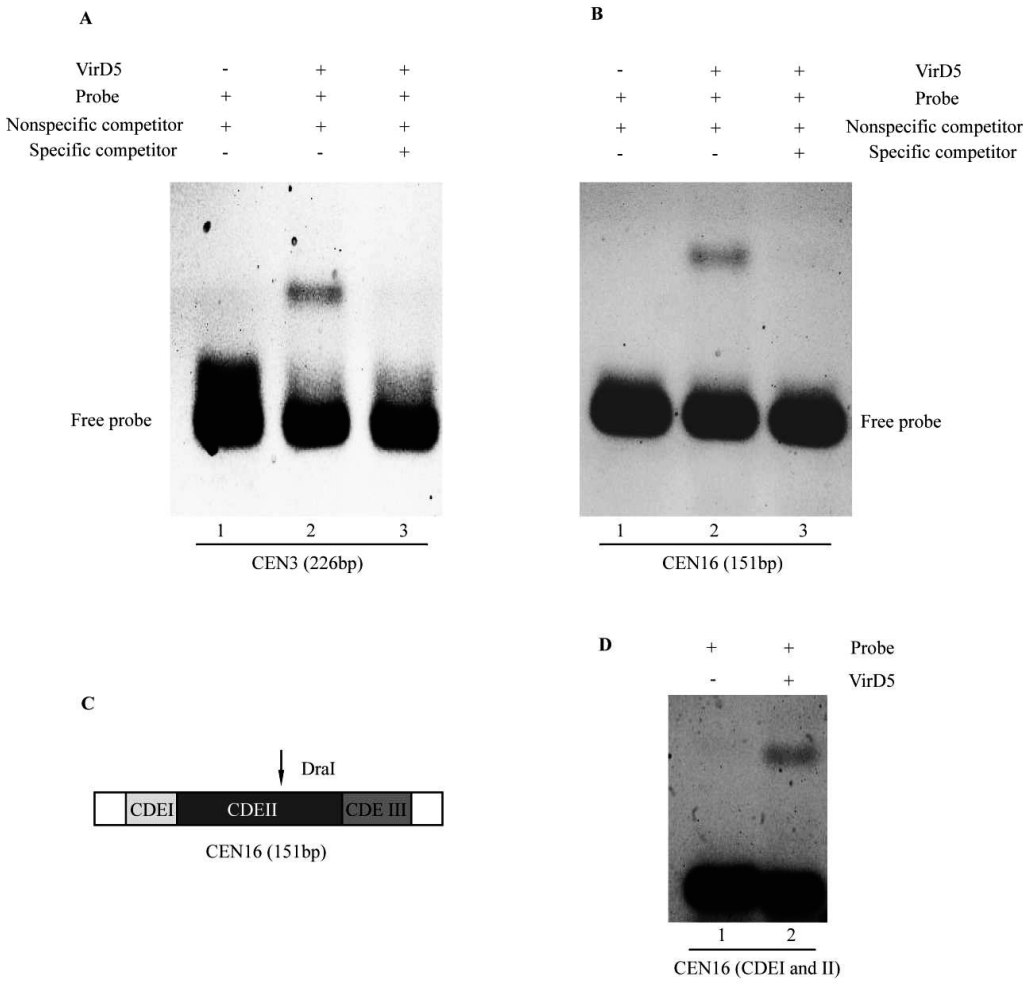


Figure 1. VirD5 binds to yeast centromeric DNA. (A-B) EMSA were performed using yeast centromeric DNA from chromosomes 3 (A) and 16 (B) as probes. His-tagged VirD5 purified from *E.coli* was incubated with probes. Non-labelled CEN3 or CEN16 was added as the specific competitor. (C) Representation of the DraI restriction site within CDEII of CEN16. (D) The left region of CEN16 containing CDEI and a part of CDEII was used for EMSA with or without the addition of His-tagged VirD5. All experiments were repeated three times.

showed two bright dots, one in the mother and the other that had moved into the daughter cell (**Figure 3B**, upper panel). In the presence of VirD5 both dots representing the position in the cells where the chromosomes are located were still close together confirming the spindle elongation defect. The two dots were either distributed over mother and daughter cell (56%) as in the absence of VirD5 or lagging in the middle of the mother cell (42%) (**Figure 3B**, middle and lower panel). These results demonstrate that VirD5 interferes with spindle microtubule elongation and entrance of the spindle into daughter cells.

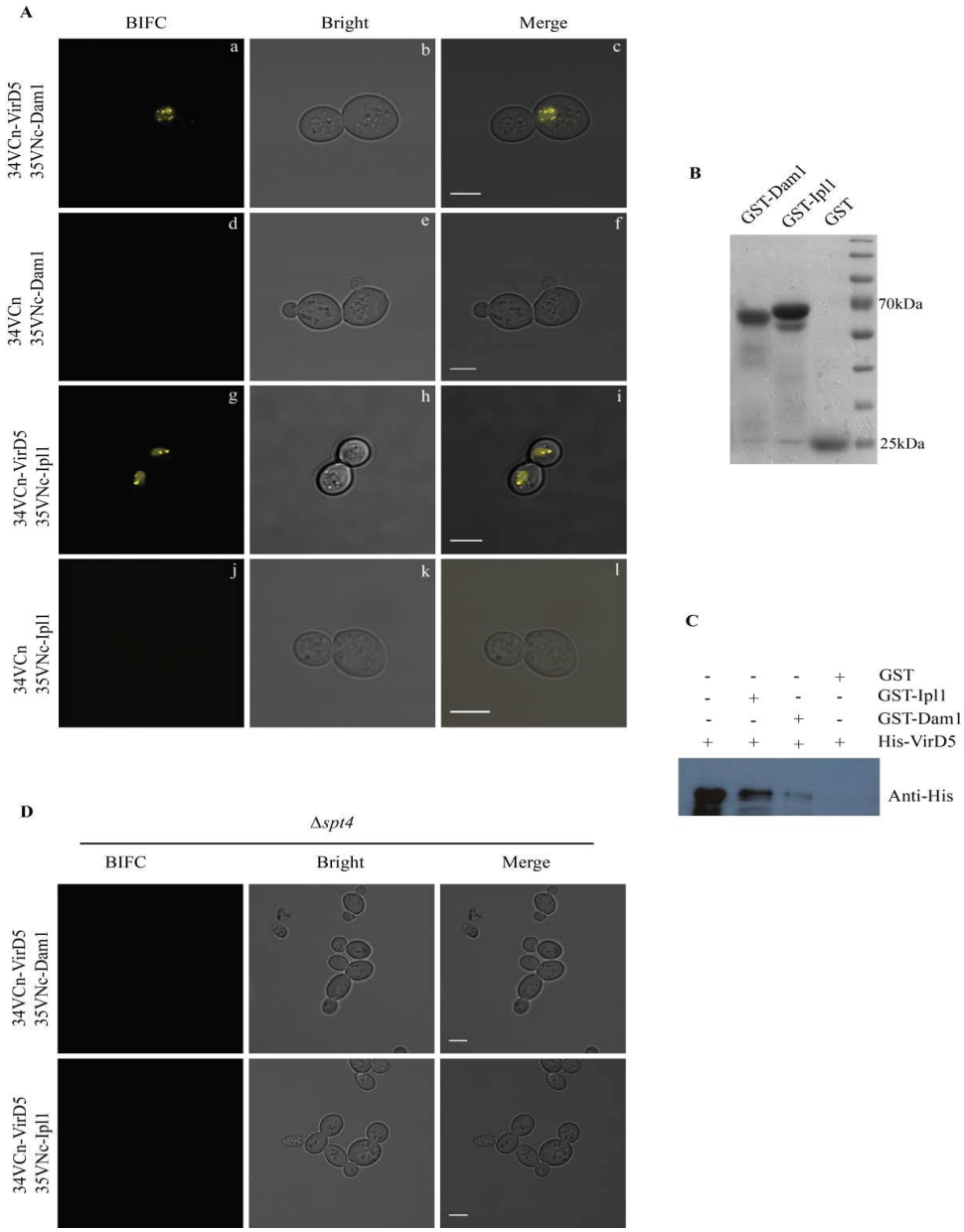


Figure 2. VirD5 physically interacts with kinetochore-associated proteins. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP (VC173) fused with the N-terminus of testing proteins. 35VNc, the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. Scale bar, 5 μm. (B) GST or GST-tagged proteins were expressed and purified from *E. coli*. (C) His-tagged VirD5 purified from *E. coli* was incubated with either empty GST or GST-Ipl1 or GST-Dam1, and after washing steps the presence of His-VirD5 was detected by anti-His antibody. (D) BIFC vector 34VCn-VirD5 either with 35VNc-Dam1 or 35VNc-Ipl1 were transformed into Δ*spt4* cells for BIFC detection. Scale bar, 5 μm.

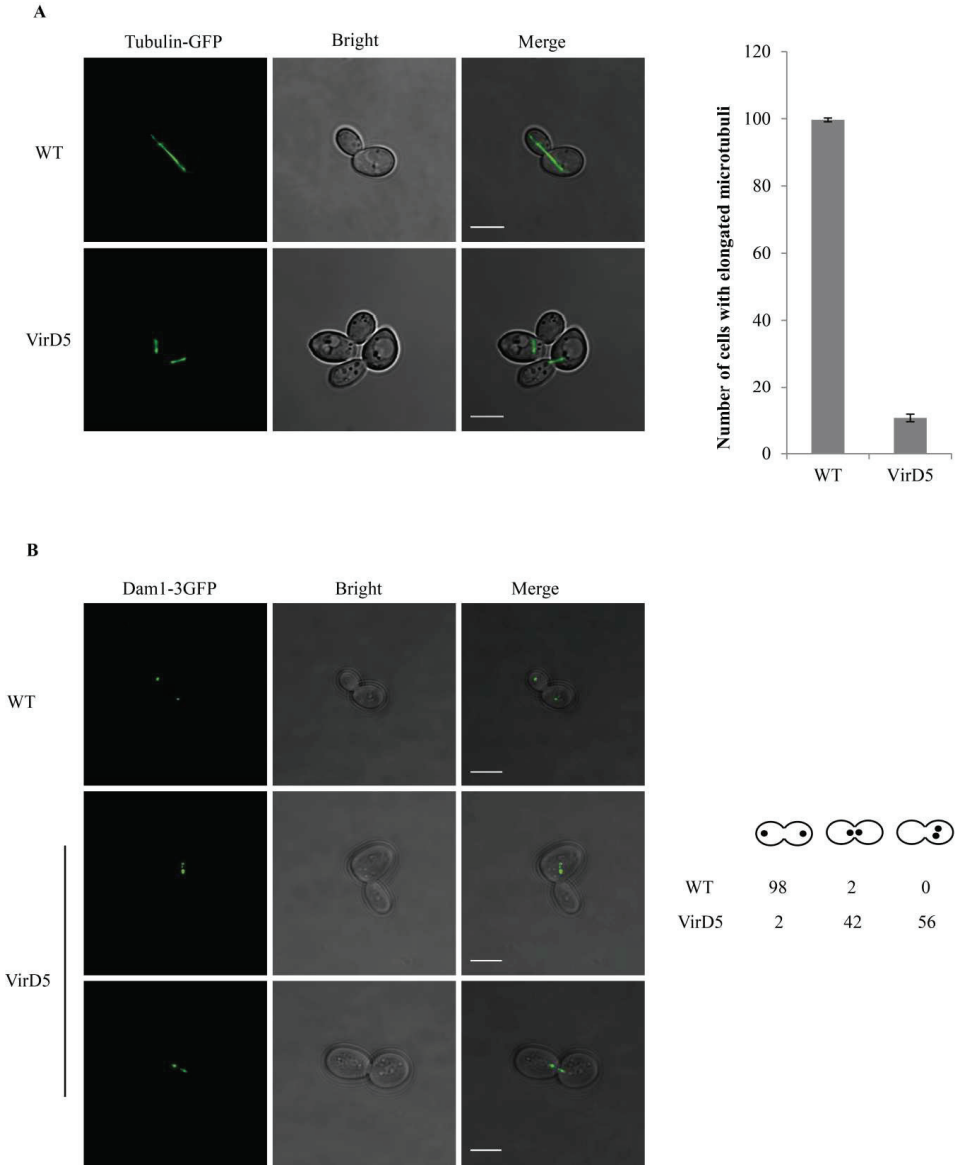


Figure 3. VirD5 disturbs the separation of sister kinetochores and spindle microtubule elongation during anaphase. (A) Tubulin-GFP marked MAS101 cells with or without the insertion of *virD5* driven by the GAL1 promoter were grown in rich medium containing glucose first and shifted to rich medium containing 2% galactose. 100 cells were counted per experiment. Scale bar, 5 μm. Error bars represent the mean ± SD from three independent experiments. (B) The kinetochore/microtubule-associated Dam1-3xGFP marked yeast cells with or without the integration of *virD5* under the control of the GAL1 promoter were grown in rich medium containing glucose first and shifted to rich medium containing 2% galactose. GFP dots were visualized under the confocal microscope. Scale bar, 5 μm. All experiments were repeated at least three times.

In the presence of VirD5 the Ipl1 kinase has an altered subcellular localization in anaphase

The spindle microtubules attach to the kinetochores and pull to separate the chromosomes destined for mother and daughter cells during mitosis. For faithful chromosome segregation, sister kinetochores must attach to spindle microtubules emanating from the two opposite poles (Foley and Kapoor, 2013; Tanaka, 2005). Incorrect attachments provoke the spindle assembly checkpoint (SAC), which arrests cells in metaphase until incorrect chromosome-microtubule attachments have been corrected (Sacristan and Kops, 2015). In yeast, the essential Aurora kinase called Ipl1, displays a dynamic subcellular localization during the cell cycle being present at the centromeres from interphase until metaphase, but at the spindle midzone during anaphase. It phosphorylates several kinetochore and microtubule-associated proteins leading to the detachment of incorrect chromosome-microtubule attachments (Biggins *et al*., 1999; Cheeseman *et al*., 2006; Keating *et al*., 2009). Earlier work in this chapter showed that VirD5 interacted with the Ipl1 kinase and is present at the centromeres/kinetochores. We therefore wondered whether the presence of VirD5 may alter the subcellular localization of Ipl1. As shown in **Figure 4A**, in wild type cells Ipl1 accumulated at the spindle midzone between the spindle pole bodies in anaphase as expected. In contrast, in cells expressing VirD5, a diffuse nuclear signal was seen, pointing to a defect in maintaining Ipl1 at the spindle midzone in anaphase.

VirD5 stimulates the kinase activity of Ipl1/Aurora on Dam1

Ipl1/Aurora kinase plays crucial roles in sensing and correcting the erroneous kinetochore-spindle microtubule attachments by phosphorylating key substrates involved in the kinetochore-spindle binding. Both loss and overexpression of the Ipl1/Aurora kinases leads to massive chromosome mis-segregation and aneuploidy in yeast cells (Chan and Botstein, 1993; Muñoz-barrera and Monje-casas, 2014*)*. Earlier work in this chapter showed that VirD5 interacts with Ipl1 and disturbs its spindle midzone localization in anaphase and thus may influence its kinase activities. In order to find out whether VirD5 may affect the kinase activity of Ipl1, we carried out an *in vitro* kinase assay using the well-known microtubule binding protein Dam1 as the substrate (Kang *et al*., 2001). GST-Dam1 expressed and purified from *E.coli*, was mixed with His-tagged Ipl1 in the presence or absence of His-tagged VirD5 in a phosphorylation buffer containing [γ-^{32}P] ATP; subsequently, the protein mixtures were separated on a SDS-PAGE gel and analyzed by autoradiography. We observed a stronger P^{32} signal on the Dam1 substrate that had been incubated with Ipl1/Aurora kinase in the presence of VirD5 (**Figure 4B**), than in control experiments in the absence of VirD5, which itself showed no kinase activity on Dam1. This suggests that VirD5 may stimulate the kinase activity of Ipl1/Aurora kinase during their interaction in yeast.

The N-terminus of VirD5 interacts with Ipl1 and Dam1

In order to find out which part of VirD5 could bind to the Ipl1 kinase and the outer kinetochore/microtubule associated protein Dam1, we performed a BIFC assay using serveral truncations of VirD5 and found that the N-terminal 505 amino acids of VirD5 (VirD5NT) gave strong interaction signals with both Ipl1 and Dam1 (**Figure 5A**). In our previous study we found that this same VirD5NT was responsible for localization at the centromeres/kinetochores and led to growth inhibition in yeast cells (**Chapter 2**). This

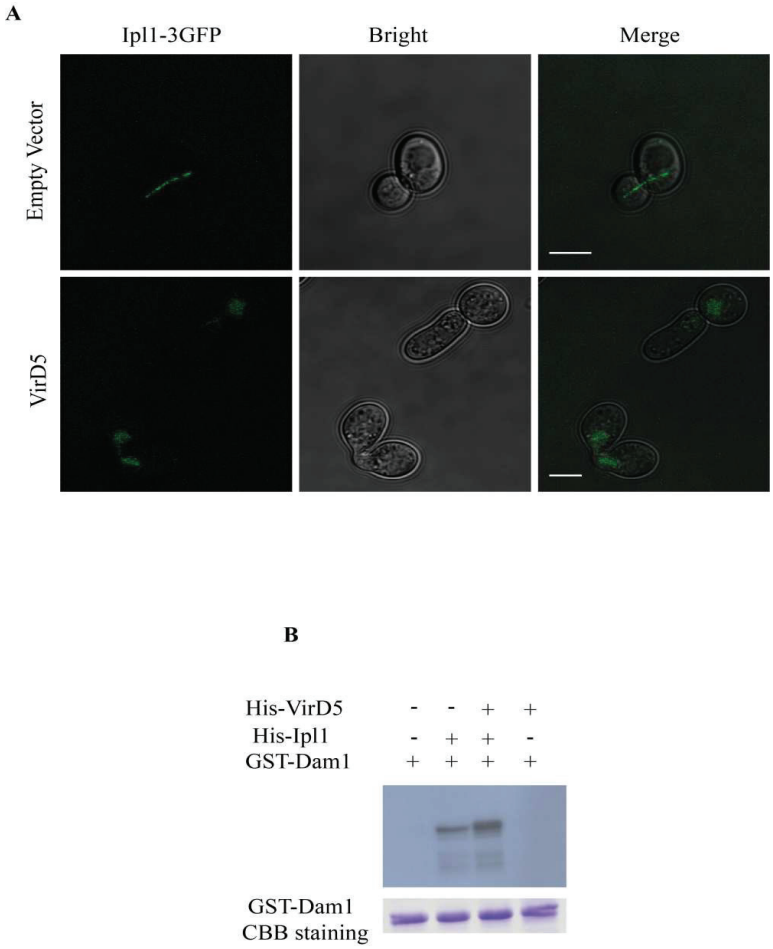


Figure 4. VirD5 stimulates the kinase activity of Ipl1. (A) Ipl1-3xGFP marked cells (YB3039) were transformed with either empty plasmid or plasmid containing *virD5* under the control of the MET25 promoter. After shifting to methionine free medium, GFP signal was observed by the confocal microscope. Scale bar, 5 μm. 100 cells per experiment were observed. (B) GST-tagged Dam1 purified from *E. coli* was incubated with His-tagged Ipl1 with or without His-tagged VirD5 protein in kinase buffer containing [γ-^{32}P] ATP. Coomassie Brilliant Blue (CBB) showed that equal amounts of GST-Dam1 were used for the kinase assay.

evoked our interest in the N-terminal part of VirD5. Further protein sequence alignment showed that VirD5NT contains 6 degenerate repeats each of which consists of around 40 amino acids (**Figure 5B**). To determine whether these repeats are all essential for the toxicity, VirD5NT and VirD5 derivatives with a deletion of each individual repeat were expressed under the control of the GAL1 promoter in yeast BY4743 cells. After induction on galacose containing medium , we found that the deletion of repeat 5 had led to a robust loss of toxicity of both VirD5NT (**Figure 5C**) and VirD5 (data not shown). In order to find out why toxicity of VirD5 lacking repeat 5 was diminished, we analyzed whether this protein still was localized at the centromers and whether it still could interact with Ipl1.

In a BIFC experiment we found that the VirD5NTΔR5 protein in contrast to VirD5 no longer gave a fluorescence signal with Ipl1 and only a weak signal with Dam1 (**Figure 6A**). This indicates that VirD5NTΔR5 no longer interacts with Ipl1 explaining its loss of toxicity. In order to find out whether this loss of interaction is due to an inability to localize at the centromeres/kinetochores we next determined the subcellular localization of VirD5NT and VirD5NTΔR5. We found that both proteins were still targeted to the centromeres/kinetochores in the nucleus (**Figure 6B**), although the numbers of fluorescent foci (1-4) were lower than seen with full length VirD5 (**Chapter 2**), indicating that targeting to the centromeres/kinetochores by VirD5NT is independent of repeat 5. Apparently, repeat 5 is important for a strong interaction with both Ipl1 and Dam1.

The toxicity of VirD5NT is suppressed by the Glc7 phosphatase and its regulatory proteins

It has been found that protein phosphatase I (Glc7 in budding yeast) can oppose the kinase activity of Ipl1/Aurora kinase by dephosphorylating the same substrates to tightly regulate the cell cycle during mitosis (Francisco, Wang, and Chan, 1994; Hsu *et al*., 2000; Pinsky, *et al*., 2006). The Glc7 activity is regulated by several regulatory (activating) subunits such as Glc8, Gip3 and Gip4 (Nigavekar *et al*., 2002; Pinsky *et al*., 2006). If the toxicity of VirD5 is indeed based on the stimulation of the kinase activity of Ipl1, overexpression of the opposing phosphatases should be able to rescue cells from toxicity. To test this, we used a truncation of VirD5 consisting of the N-terminal 505 amino acids (VirD5NT) which was shown to interact with the Ipl1 kinase (**Figure 5A**) to carry out a suppressor assay. As shown in **Figure 6C**, VirD5NT inhibited the growth of yeast cells, but this growth inhibition was indeed suppressed by overexpression of Glc7 or any of its three activating regulatory proteins. This indicates that indeed the increased kinase activity of Ipl1 underlies the toxicity of VirD5NT.

VirD5 induces chromosome mis-segregation

In our previous study we found that VirD5 induces aneuploidy which is a consequence of chromosome mis-segregation (**Chapter 2**). To confirm chromosome mis-segregation caused by VirD5 directly, we integrated *virD5* driven by the GAL1 promoter into the haploid yeast strain Y716 (Meyer *et al*., 2013). This strain expresses a GFP-LacI fusion protein that binds to a 256 tandem repeat *lac*O operator array integrated into chromosome I of this yeast strain, thus marking chromosome I with a green fluorescent bright dot inside the nucleus that can be seen under the microscope. In the presence of galactose, cells lacking VirD5 showed equal chromosome I segregation (**Figure 7**, a-c), whereas approximately 97% of the cells expressing VirD5 displayed an erroneous distribution of chromosome I over the daughter cells (**Figure 7**, d-l). We found three classes of mis-segregation: the major class represents mother cells with two chromosomes I, and daughter cells lacking chromosome I (**Figure 7**, g-i), the second class comprises mother cells with chromosome I, and daughters lacking chromosome I (**Figure 7**, d-f), and the last class of cells represents pairs of cells with in total more than two bright GFP dots (**Figure 7**, j-l). VirD5NT was shown to affect mitosis and inhibit yeast growth (**Chapter 2**).

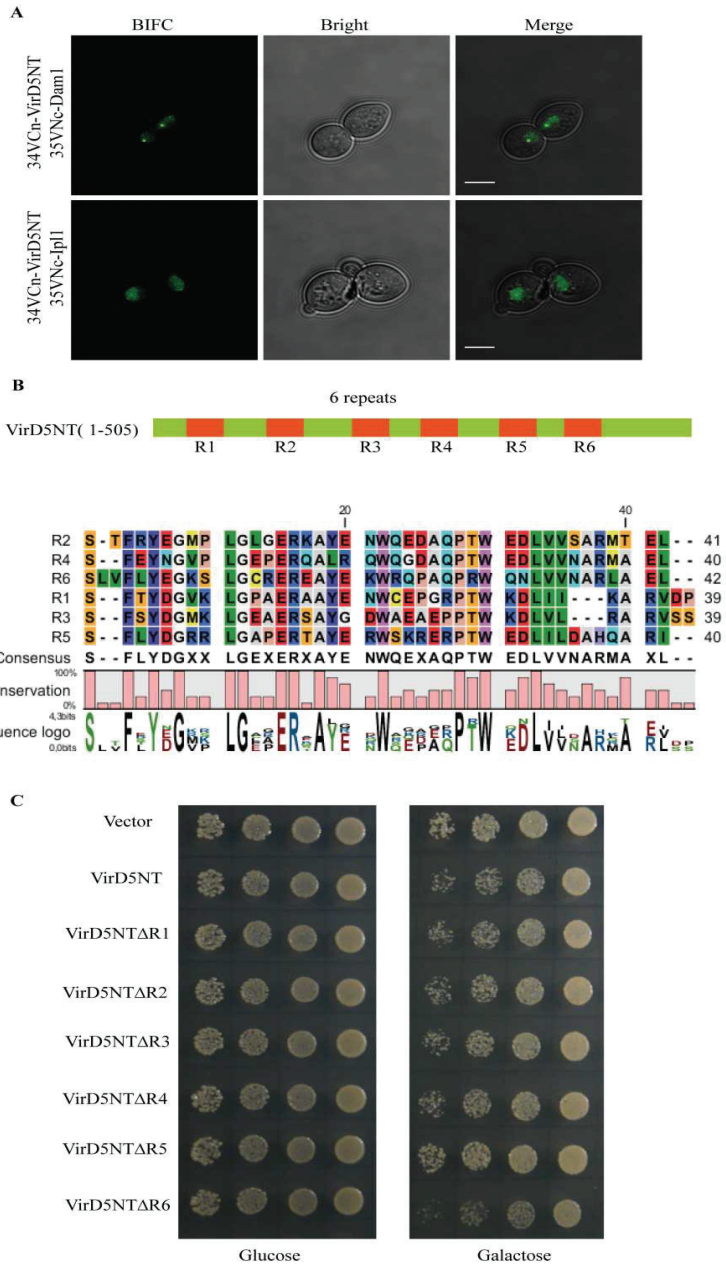


Figure 5. The N-terminus of VirD5 interacts with Ipl1 and Dam1. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP fragment (VC173) fused with the N-terminus of testing proteins. 35VNc, the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein. Scale bar, 5 μm. (B) Sequence alignment of the N-terminal repeats of VirD5. (C) Empty vector or derivatives expressing either VirD5NT or VirD5NT lacking an individual repeat driven by the GAL1 promoter were transformed into diploid yeast cells (BY4743). Transformants were serially diluted and spotted onto selection medium containing either glucose or galactose.

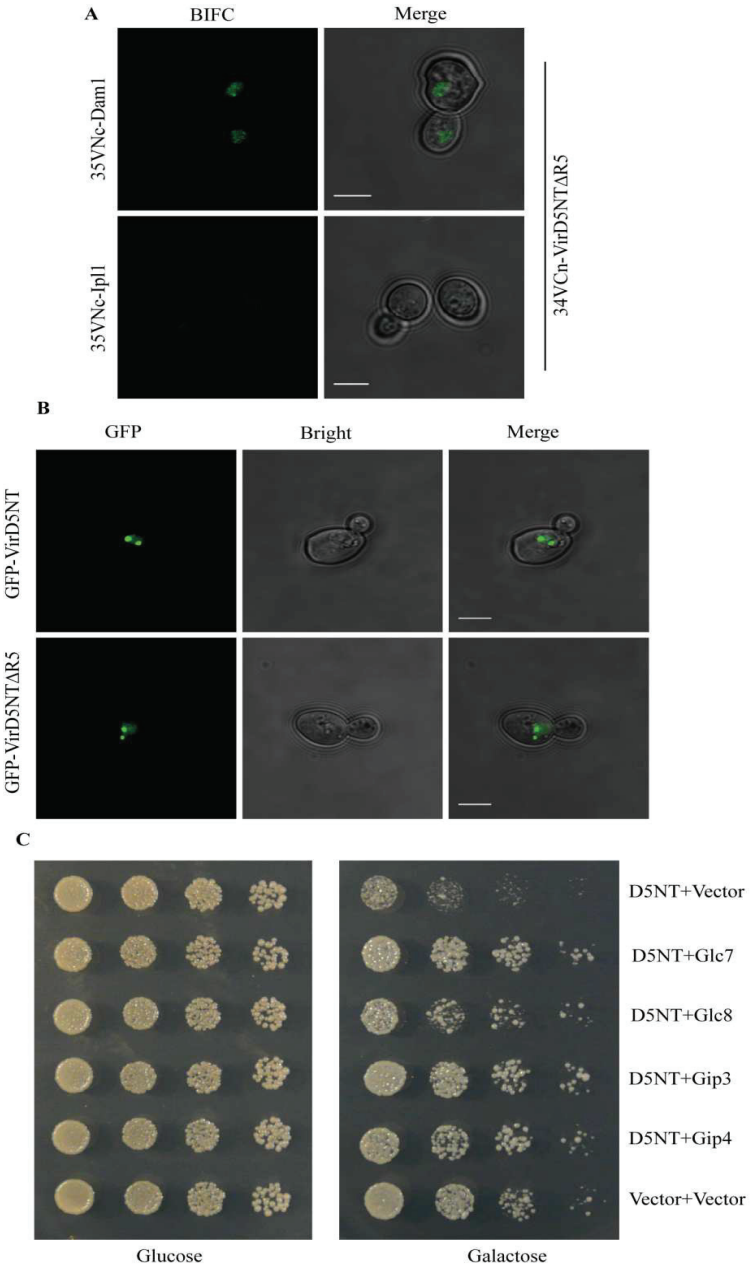
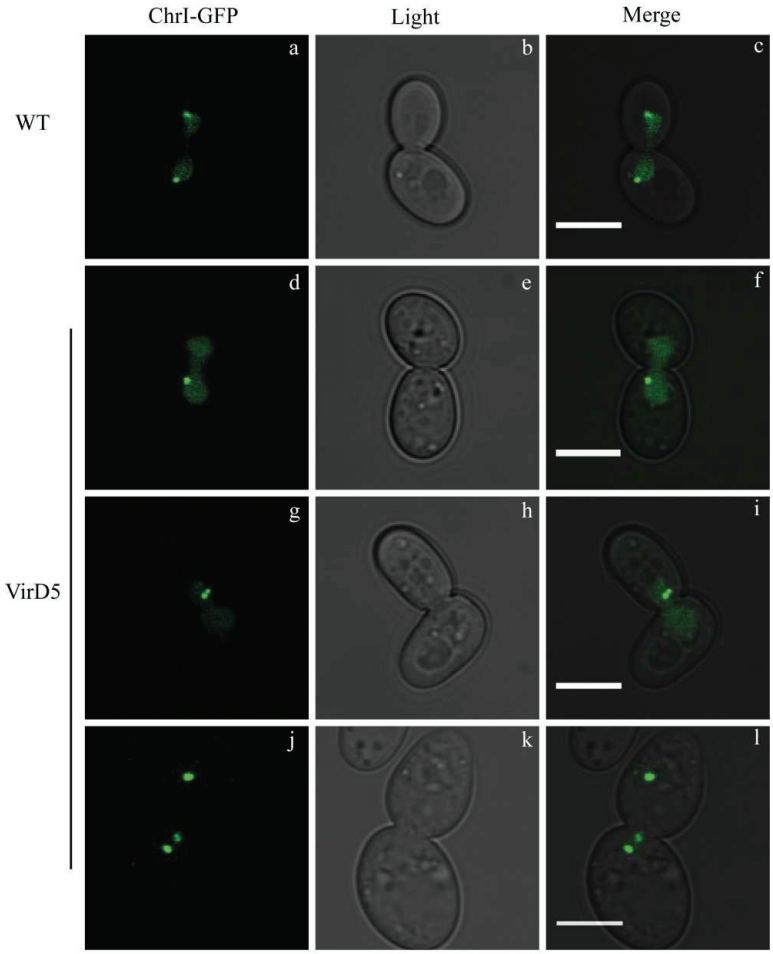


Figure 6. The toxicity of VirD5NT is suppressed by different phosphatases. (A) Yeast cells were transformed with BIFC vectors. Scale bar, 5 μm. (B) Yeast cells were transformed with plasmid encoding either GFP-VirD5NT or GFP-VirD5NTΔR5. Scale bar, 5 μm. (C) Plasmid containing *virD5NT* driven by the GAL1 promoter was cotransformed into haploid BY4741 cells either with a high-copy empty plasmid or the same plasmid expressing phosphatase Glc7 or its activators from their own promoters and terminators. Transformants were serially diluted and spotted onto selection medium containing either glucose or galactose. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein.



WT	100	0	0	0
VirD5	3	42	46	9
VirD5NT	72	10	15	3

Figure 7. VirD5 disturbs chromosome segregation. The yeast strain Y716 contains a 256 repeat LacO array in Chromosome I and expresses GFP-LacI. Binding of GFP-LacI to the LacO array allows the visualization of a GFP dot. Wild type cells or cells with integration of either *virD5* or *virD5NT-3xNLS* under the control of the GAL1 promoter were cultured in rich medium containing glucose first, and were shifted to galactose containing rich medium. 100 mitotic cells from each experiment were observed. Scale bar, 5 μm.

We thus wondered whether VirD5NT was responsible for chromosome mis-segregation. To this end we examined the distribution of chromosome I dots in cells (Y716) expressing VirD5NT and found that approximately 30% of cells expressing VirD5NT displayed chromosome mis-segregation (**Figure 7**).These results confirm that VirD5 disturbs accurate chromosome segregation and consequently results in the generation of aneuploidy. Moreover, they indicate that VirD5NT is capable of inducing chromosome mis-segregation, but that the full length protein is much more effective.

VirD5 induces DNA damage

Previously we found that chromosomes isolated from cells expressing VirD5 cells were prone to breakage and fragmentation as visualized as a massive loss of clear chromosome bands on CHEF gels (**Chapter 2**). To find more direct evidence for the presence of chromosomal DNA breaks in cells expressing VirD5, we expressed VirD5 under the control of the GAL1 promoter in Rad52-GFP marked yeast cells. Visualization of foci of Rad52, the key protein involved in homologous DNA repair, allows to count the number of sites where DNA double-stranded breaks are being repaired. Using this strain we found that over 90% of the cells expressing VirD5 displayed green dots representing DNA repair foci in the nucleus, while only very few cells lacking VirD5 (~5%) showed a single DNA repair focus (**Figure 8A** and **B**).

We found that VirD5NT increased the frequency of chromosome mis-segregation (**Figure 7**), but to a lesser extent than the full length VirD5 protein. In order to find out whether VirD5NT would be sufficient for the induction of DNA breaks, we counted the number of Rad52-GFP foci in cells with a chromosomally integrated construct encoding VirD5NT. As can be seen in **Figure 8B**, cells expressing VirD5NT showed a more than two-fold higher number of DNA repair foci compared with wild type cells. We next determined whether this increased DNA damage caused by VirD5NT could be strong enough to trigger the massive chromosomal fragmentation seen previously with chromosomal preparations from cells expressing VirD5. To this end chromosomes from yeast cells with a chromosomally integrated plasmid encoding either GFP or GFP-VirD5NT under the control of the MET25 promoter, or from cells with a high-copy empty plasmid or plasmid containing *virD5NT* driven by the GAL1 promoter, were isolated and separated in a CHEF gel. The N-terminal part of VirD5 did not generate the massive chromosomal fragmentation seen after expression of the full length protein (**Figure 8C**), although the presence of VirD5NT increased the numbers of Rad52-GFP foci in yeast cells (**Figure 8B**). These results strongly suggest that VirD5 causes DNA breaks, but this property requires both the N-terminal and the C-terminal parts of VirD5.

Discussion

In this study, we have shown that VirD5 directly binds to the centromeric DNA (**Figure 1**) which confirmed our previous bioinformatics prediction showing that VirD5 contains several DNA binding motifs (Schrammeijer *et al.*, 2001). Whether this DNA binding activity has any biological meaning is unclear at the moment, but in any case is not strong enough to bring VirD5 to the centromeres as in the absence of Spt4 the protein is no longer localized at the centromeres (**Chapter 2**).

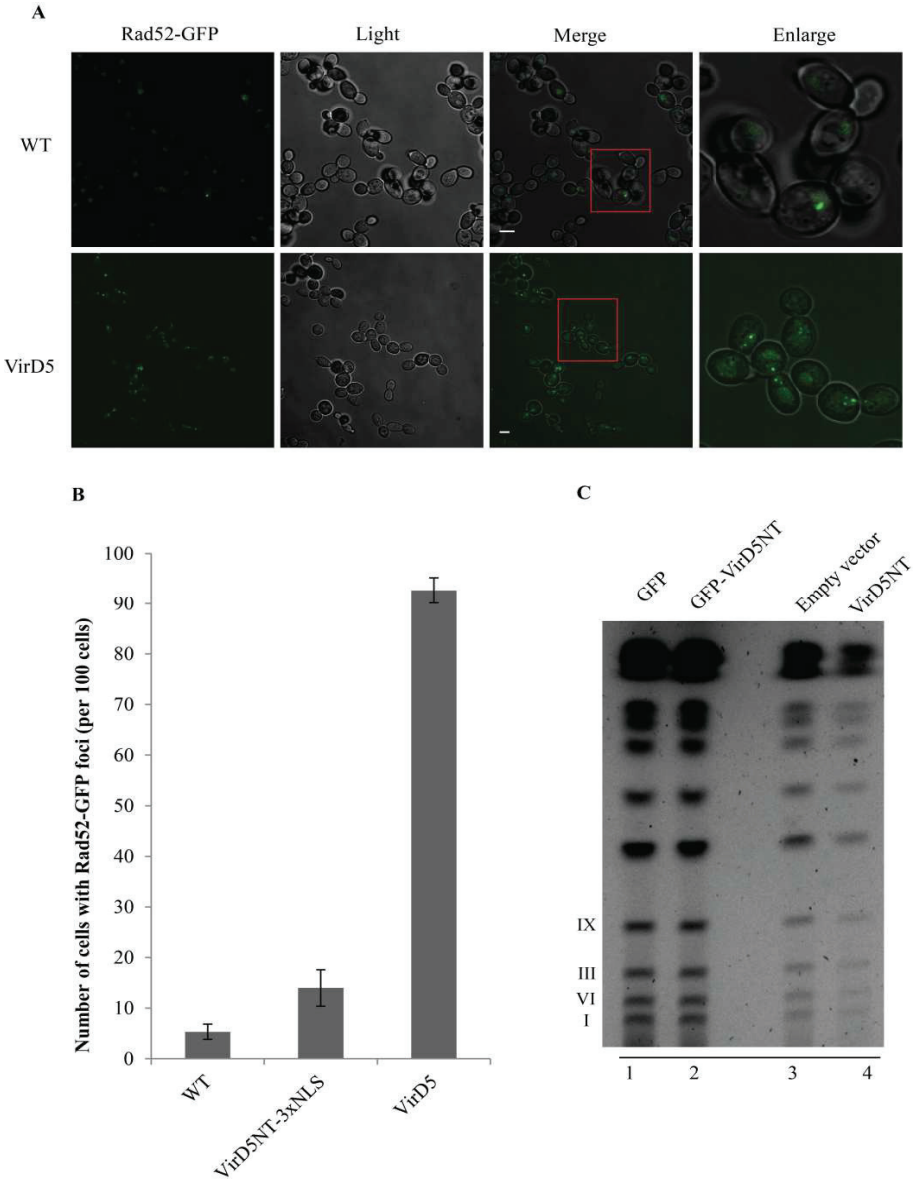


Figure 8. VirD5 causes massive DNA damage. (A-B) A Rad52-GFP marked strain with or without the integration of either the *virD5* or *virD5NT-3xNLS* gene driven by the GAL1 promoter was cultured in rich medium containing glucose, followed by a shift to rich medium containing galactose. The DNA repair foci were observed and counted under the confocal microscope. Scale bar, 5 μm. Error bars represent the mean ± SD from three independent experiments. (C) Chromosomes from yeast (BY4743-H2A-CFP) with a chromosomally integrated construct encoding either GFP (1) or GFP-VirD5NT (2), or from BY4743 transformed either with a high-copy empty plasmid (3) or a plasmid encoding VirD5NT driven by the GAL1 promoter (4), were isolated and separated in a CHEF gel. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein.

The presence of VirD5 did not disturb the co-localization pattern of the centromeres/kinetochores in the cell as both in absence and in presence of VirD5 the kinetochore protein Dam1 showed the same single dots in the nucleus as visualized after GFP fusion (**Figure 3B**). However, VirD5 interfered with spindle elongation, positioning at the bud and entrance in the daughter cell in anaphase (**Figure 3A**), thus preventing the faithful sister-chromatid separation over the daughter cells during mitosis (**Figure 3B**). Also the fast elongation of the microtubules seen in the absence was no longer seen in the presence of VirD5 indicating that cells with VirD5 are arrested by the spindle attachment checkpoint (SAC) or otherwise suffer from an inhibitory effect on spindle microtubule elongation. The spindle microtubules only elongate after proper amphitelic attachment of all the kinetochores and then separate the chromatids from each other by pulling them to the opposite spindle poles. Erroneous attachment of any of the kinetochores activates the Ipl1/Aurora kinase, which phosphorylates outer kinetochore proteins of the Ndc80 complex and proteins of the Dam1 complex associated with the microtubules, which leads to detachment (Cheeseman *et al*., 2002; DeLuca *et al*., 2011; Saurin *et al*., 2011). If any of the kinetochores is not attached to a microtubule the SAC is activated and only inactivated after proper attachment of all the kinetochores is realized (Sacristan and Kops, 2015). We found that VirD5, when present through interaction with Spt4 at the kinetochore, could interact with both the Dam1 complex and Ipl1/Aurora kinase (**Figure 2A** and **C**). The yeast Dam1 complex encircles the plus ends of microtubules (Miranda *et al*., 2005), and is essential for binding of the microtubules to the kinetochores through a direct interaction with the outer kinetochore protein Ndc80 (Lampert *et al*., 2013; Tien *et al*., 2010). The human Ska1 protein complex was recently identified as having a similar structure and function as the Dam1 complex in facilitating microtubule movement (Welburn *et al*., 2009). Irregular expression of Aurora kinases causes chromosome mis-segregation and tumor formation (Demidov *et al*., 2014; Katayama *et al*., 2003; Vader and Lens, 2008).

We found that VirD5 could interact *in vivo* at the kinetochores with the Ipl1/Aurora kinase (**Figure 2A** and **C**) and could stimulate its kinase activity and phosphorylation of the substrate Dam1 *in vitro* (**Figure 4B**). This explains why VirD5 and VirD5NT which interacts with Ipl1 and Dam1 (**Figure 5A**) cause chromosome mis-segregation and aneuploidy formation (**Figure 7**). In line with this, the toxicity of VirD5NT could be suppressed by overexpression of the Glc7 phosphatase or its activation proteins, which dampens the hyperactivity of Ipl1 due to the presence of VirD5NT (**Figure 6C**). The Glc7 phosphatase is involved in dephosphorylation of proteins phosphorylated by the Ipl1/Aurora kinase (Francisco, Wang, and Chan, 1994; Hsu *et al*., 2000; Pinsky, *et al*., 2006). We have also observed that VirD5 physically interacts with other Aurora kinases from both human and plant cells (**Chapter 4** and **5**), and found the same disturbed chromosome segregation in those cells.

It has been shown that chromosome mis-segregation can lead to DNA breaks (Janssen *et al*., 2011). In the presence of VirD5NT cells mis-segregated their chromosomes (**Figure 7**) and had more DNA damage than control cells (**Figure 8B**). This DNA damage associated with the presence of VirD5NT may thus be due to chromosome mis-segregation. The full length VirD5 protein is much more toxic than VirD5NT and this may be due to the fact that the presence of full length VirD5 generates massive DNA damage and chromosomal

fragmentation (**Figure 8C**, **Chapter 2**). This indicates that the C-terminal part of VirD5 (VirD5CT) may reinforce the biological activities of the N-terminal part or contribute other independent toxic (DNA damaging) activities. Indeed we have found that the C-terminal part of VirD5 on its own also triggers massive DNA damage (our unpublished results). Both of these biological activities of VirD5 may augment the tumor-inducing capabilities of *Agrobacterium*. The chromosomal mis-segregation may contribute to the evolution of fast-growing tumor cells and the DNA breaks induced may act as entry points for integration of the T-DNA.

Materials and Methods

EMSA assay

5' Cy5 labeled primers CEN3FW and CEN3REV, CEN16FW and CEN16REV (**Table 1**) were used to amplify yeast (BY4743) centromeric DNA from chromosomes 3 (CEN3) and 16 (CEN16), respectively. Primers with the same sequences but lacking the 5' Cy5 label were used to obtain the same unlabeled fragments from CEN3 and CEN16 to be used as specific competitor fragments. PCR products were purified (Zymo research D4002) and sent for sequencing. His-tagged VirD5 protein was expressed in *E.coli* strain Rosette2PLySs and purified with Ni-NTA Agarose (QIAGEN: Cat 30230). The EMSA assay was carried out according to Hellman and Fried (2007) with some modifications. The EMSA reaction contained 4µL 5x EMSA buffer (50 mM HEPES pH 7.9, 375 mM KCl, 12.5 mM MgCl2, 0.5 mM EDTA, 5 mM DTT, and 15% Ficoll), 1 µL 1 mg/ml PolydI/dC (Sigma cat: 81349), 1µL 10 mg/ml BSA, 7 µL purified His-tagged VirD5 protein, 6 ng Cy5 labeled probe, and water to a final volume of 20 µL. The mixture was incubated for 30 minutes at room temperature. The reaction was stopped by loading to a 2% agarose gel for electrophoresis in 0.5xTBE buffer at the voltage of 50 V for 7 hours in the cold room, followed by scanning with Perkin Elmer ProExpress 2D Proteomic Imaging System (American Laboratory Trading ALT).

BIFC assay

The pUG34VCn-VirD5 plasmid (or pUG34VCn-VirD5NT) was transformed either with pUG35VNc-Spt4 or pUG35VNc-Dam1 or pUG35VNc-Ipl1 into wild type yeast cells. Transformants were grown at 30 oC on solid MY medium containing methionine to inhibit the expression of VirD5. After 3 days, colonies were transferred to MY liquid medium containing methionine. Overnight cultures washed twice with sterilized water were transferred into new flasks containing MY medium lacking methionine to induce the expression of VirD5. After induction for 1 hour, cells were harvested for BIFC signal visualization using a 63xoil objective on the Zeiss Imager confocal microscope. Images were processed with ImageJ (ImageJ National Institutes of Health).). Plasmids and yeast strains used in this study are listed in **Table 3** and **4**, respectively.

In vitro kinase assay

His-tagged Ipl1 and His-tagged VirD5 were expressed and purified with Ni-NTA Agarose (QIAGEN: Cat 30230). GST-tagged Dam1 protein was expressed in *E.coli* strain Rosette2PLySs. 50 ml cell cultures were centrifuged and resuspended in 1 ml lysis buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA, 1% Triton X-100). After

sonication and centrifugation, the supernatants were stored at -80 °C. The Kinase assay was performed (Keating *et al.*, 2009) by incubating GST-Dam1 (10 µL supernatant from 1ml lysate)-bound glutathione beads, 1 µL purified His-Ipl1 and 8 µL purified His-VirD5 in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) β-mercaptoethanol, 0.1 mM EGTA, 10 mM $MgCl_2$, 100 µM ATP and 1 μCi of [γ-^{32}P] ATP for 1 hour at 30 °C. The reaction was stopped by adding 5 µL 4 x sample buffer and boiling for 10 minutes and the mixture was loaded to a 10% SDS-PAGE gel and processed for autoradiography or CBB staining to confirm the equivalent loading of GST-Dam1 protein.

Pull-down assay

GST, GST-Dam1 and GST-Ipl1 were expressed in *E.coli* strain Rosette2PLySs. Equal amounts of the GST-tagged proteins were immobilized on Glutathione HiCap Matrix (Qiagen, 30900) for 2 hours at room temperature, followed by a 3 times washing step with washing buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA). The beads were incubated with purified His-tagged VirD5 protein in binding buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 0.1% Triton X-100) for 2 hours at room temperature. After 3 times washing with buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40), samples were mixed with 20 µL 4x sample buffer and boiled for 10 minutes, followed by centrifugation for 2 minutes at 2000 rpm. Supernatants were loaded to a 10% SDS-PAGE gel for electrophoresis. The presence of the His-tagged VirD5 protein was detected with Anti-His HRP antibodies (Santa Cruz Biotechnology, sc-8036 HRP) by Western Blot analysis.

Chromosome segregation assay

Y716 strain (a gift from Dr Dean Dawson) contains a GFP-LacI and a 256 repeat LacO array integrated in chromosome I. Enrichment of GFP-LacI to the LacO repeats allows the visualization of chromosome I as GFP dot. Wild type and cells integrated with *virD5* or *virD5NT-3NLS* driven by the GAL1 promoter at the URA3 locus were cultured in rich medium containing glucose. Overnight cultured cells were diluted to an OD_{620} of 0.1 and recultured in rich medium containing 2% raffinose and 2% galactose for additional 6 hours. GFP dot signal was observed via a 63xOil objective on the Zeiss Imager confocal. 100 anaphase cells were analyzed for each experiment.

Separation of chromosomes on CHEF gels

Intact yeast chromosomes were isolated in agarose plugs as described from the CHEF kit (Bio-Rad, 170-3591). $6x10^8$ cells overnight cultured yeast cells were washed twice with 0.1 M EDTA (pH 7.5) and resuspended in 630 µL suspension buffer. The suspension mixed with 370 µL 2% low-melt agarose was used to make plugs (10 plugs) for CHEF. Plugs were placed in a 1% agarose gel and sealed with liquid agarose. Electrophoresis was carried out in 0.5xTBE at 14 °C for 24 hours with an initial switch time of 60 s and a final time of 90 s at 200 V. The separated chromosomes were stained with ethidium bromide.

Acknowledgements

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Table 1. Primers used in this study.

Name	Sequence (5'-3')
Bub2TF	AACGACGTAAAGGTAAAAGAAACAACAGACTTTTAAACTTGTTAA CTTTTGCCGTACGCTGCAGGTCGAC
Bub2TR	CGTTGTAGAATTAAACGATAAAATATAATATTTCTTCACATAGTTT ACGGTATCGATGAATTCGAGCTCG
CEN3FW	CAATATGGAAAATCCACAGAAAGCTATTC
CEN3REV	CCACCAGTAAACGTTTCATATATCCATTC
CEN16FW	CATGGTAGTGATCACAAATAGATCACA
CEN16REV	CAACTGAATAATATTTCTATTTTCGGA
Dam1mycF	CTGTTGCAAAGAAAACTGAAAAAAAAATAAATACAAGGCCCCCCT TCAGACGTACGCTGCAGGTCGAC
Dam1mycR	GCGATATATTTTGTGAGGAGGATAATTCTTTGGTTGGGTTGGGCGT AGTCAATCGATGAATTCGAGCTCG
Dam1FW	CATGCTAGCCATGAGCGAAGATAAAGCTAAAT
Dam1REV	ACGCGTCGACGTCTGAAGGGGGGCCTTGTA
FlagF2	ACGCGTCGACATGGACTACAAGGACGACGACGATAAG
Ipl1FW	GGACTAGTCATGCAACGCAATAGTTTAGT
Ipl1REV	ACGCGTCGACGTAACCGCTTATTTTCCCAAAAGG
Ipl1HisFW	CCATCGATATGCAACGCAATAGTTTAG
Ipl1HisREV	CCCCCCGGGCTATAACCGCTTATTTTCCC
Ipl1TF	AATGCATCCTTGGATACTAAGAAACAAGCCCTTTTGGGAAAATAA GCGGTTACGTACGCTGCAGGTCGAC
Ipl1TR	GGGAGTGATTAATAGTGCCCTTCAAACGATTCTGTCATACTTTAAT TCTAATCGATGAATTCGAGCTCG
pMVHisVirD5FW	GGACTAGTTCACGCTGGGCGTAACCACCA
pMVHisVirD5REV	ACGCGTCGACATTAAAGCCTTCGAGCGTCCC
R1F	ACGCGTCGACGACGATGTGATCTGGCTC
R1R	CCGCTCGAGCCGGCCCGGTTCGCACC
R2F	ACGCGTCGACGGCCGTCCACACGGGATT
R2R	CCGCTCGAGCTGAGCGTCCTCTTGCCA
R3F	ACGCGTCGACAATGACTCTGCGTGGCTTT
R3R	CCGCTCGAGTGGCTCCGCCTCGGCCCAG
R4F	ACGCGTCGACTGCCATGCTGGTTGGATT
R4R	CCGCTCGAGCTGAGCGTCTCCTTGCCA
R5F	ACGCGTCGACGAAAGTGATGCTGTTACG
R5R	CCGCTCGAGGCGTTCCCTCTTACTCCAG
R6F	ACGCGTCGACGATCCCTCAGTCTGGATTG
R6R	CCGCTCGAGTTGAGCTGGCTGCCGCCAT
Rad52F2	AGAGAAGTTGGAAGACCAAAGATCAATCCCCTGCATGCACGCAAG CCTACTCGTACGCTGCAGGTCGAC
Rad52R2	AGTAATAAATAATGATGCAAATTTTTTATTTGTTTCGGCCAGGAAG CGTTTCAATCGATGAATTCGAGCTCG
Spt4TF2	ACCTGGCCACATTCAGTTTGGCAAAAGCGAACGAGGTACAGTGTA AGAGCGTACGCTGCAGGTCGAC

Spt4TR2	CATGTGATATCAGAACGGAAGGTTTTACTCAACTTGACTGCCATCC CTCGGATCGATGAATTCGAGCTCG
SPT4FW	AAAGCGGCCGCTCCAATTTACGTGAAGTAGAT
SPT4REV	CCCCCCGGGACCTTTTTTTTCTAATGAAAGTC
VirD5#1	CCGCCCGGGGATGACAGGAAAG
VirD5#1-2	CGCCTGCAGGACGGGATCGCTG
VirD5#3-2	CGCCTGCAGCGGCGGAACAAGGAC
VirD5#4-2	CGCCTGCAGTCAGCGTTTAAAC
VirD5#9	CCGCCCGGGGGATAAAAACGAAGCCCC
VirD5#10-2	AAAGCGGCCGCTCAGCGTTTAAACGC
VirD5#21	CCATCGATATGACAGGAAAGTCG
VirD5#21-2	CCCCCCGGGTCAGCGTTTAAAC
VirD5#23	GGACTAGTATGACAGGAAAGTCG
VirD5#23-2	ACGCGTCGACTCAGCGTTTAAAC
VirD5#28	CCGCCCGGGATGACAGGAAAGTCG
VirD5#33	AAAGCGGCCGCAAACAGGAAAGTCGAAAGTTC
VirD5#34	AAAGCGGCCGCAACAGGCTGATGCCTCGTTTG
VirD5#38	GGACTAGTATGACAGGAAAGTCGAAAGTTCAC
VirD5#41	CCGCTCGAGTCAGACGGGATCGCTG
VirD5#52	TGCTCTAGATTAGCGTTTAAACGCTTTGTC
VirD5#63	ACGCGTCGACGGAGATATACCATGGGC
VirD5N349R	CCGCTCGAGGATGAGATCTTCCCAGGTC
VirD5N354F	ACGCGTCGACCAGGCCAGGATTGAAAGTG
VirD5N355F	ACGCGTCGACGCCAGGATTGAAAGTGATG

Table 2. Candidate centromeric-kinetochore proteins tested for interaction with VirD5 in BIFC.

Protein name	Function-localization	Interaction
Spt4	Kinetochore, telomere	Y
Ndc10	Inner kinetochore	N
Cep3	Inner kinetochore	N
Ctf13	Inner kinetochore	N
Cse4	Inner kinetochore	N
Mif2	Inner kinetochore	N
Cbf1	Inner kinetochore	N
Mad2	SAC checkpoint	N
Ipl1	MT-Kinetochore regulatory aurora kinase	Y
Dam1	Microtubule-associated protein	Y
Ndc80	Outer kinetochore	N
Scc1	Cohesin	N
Esp1	Separase	N
Skp1	Inner kinetochore	N

Table 3. Plasmids used in this study.

Name	Description	Sources/ references
pMVHis	High-copy vector with a GAL1 promoter and a *URA3* marker.	(van Hemert, *et al*., 2003)
pMVHis-VirD5	VirD5 (XmaI-NotI) was inserted into pMVHis.	This study
pMVHisVirD5ΔR1	VirD5ΔR1 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR2	VirD5ΔR2 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR3	VirD5ΔR3 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR4	VirD5ΔR4 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR5	VirD5ΔR5 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR6	VirD5ΔR6 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-202)	VirD5 (1-202) (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505)	VirD5 (1-505) (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR1	VirD5 (1-505) ΔR1 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR2	VirD5 (1-505) ΔR2 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR3	VirD5 (1-505) ΔR3 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR4	VirD5 (1-505) ΔR4 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR5	VirD5 (1-505) ΔR5 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR6	VirD5 (1-505) ΔR6 (XmaI-XhoI) was inserted into pMVHis.	This study
pRS315	Single-copy yeast plasmid with a *LEU2* marker.	(Sikorski and Hieter, 1989)
pRS315-Spt4	Spt4 including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pUG34GFP	Single-copy plasmid with a *HIS3* maker for a fusion with the C-terminus of GFP driven by the MET25 promoter.	(Sakalis, 2013)
pUG34-GFP-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34GFP.	This study
pUG34-GFP-VirD5NT (1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34GFP.	This study
pUG34-2xGFP-VirD5NT (1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34-2xGFP.	This study
pUG34-GFP-VirD5NT (521-833)	VirD5 (521-833) (SpeI-SalI) was inserted into pUG34GFP.	This study
pET16H	pBR322 base plasmid with an N-terminal10xHis tag under the control of the T7 promoter.	Novagen
pET16H-VirD5	VirD5 (ClaI-XmaI) was inserted into pET16H.	This study
pET16H-Ipl1	Ipl1 (ClaI-XmaI) was inserted into pET16H.	This study
pGEX-KG	pMB1 based plasmid with an N-terminal GST tag under the control of the TAC promoter.	(Guan and Dixon, 1991)
pGEX-KG-Ipl1	Ipl1 (SpeI-SalI) was inserted into (XbaI-SalI) of pGEX-KG.	This study
pGEX-KG-Dam1	Dam1 (NheI-SalI) was inserted into(XbaI-SalI) of pGEX-KG.	This study

pUG34VCn	Single-copy plasmid with an N-terminal fusion with the C-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG34VCn-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34VCn.	This study
pUG34VCn-VirD5(1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34VCn.	This study
pUG34VCn-VirD5(1-505) ΔR5	VirD5 (1-505) ΔR5 (SpeI-XhoI) was inserted into pUG34VCn.	This study
pUG35VNc	Single-copy plasmid with an C-terminal fusion with the N-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG35VNc-Ipl1	Ipl1 (SpeI-SalI) was inserted into pUG35VNc.	This study
pUG35VNc-Dam1	Dam1 (NheI-SalI) was inserted into (SpeI-SalI) of pUG35VNc.	This study
pRS425	High-copy plasmid with a *LEU2* marker.	(Christianson *et al*., 1992)
pRS425-pGAL1-VirD5(1-505)	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5(1-505) as template was inserted into SpeI-SalI of pRS425.	This study
pRS425-Glc7	Glc7 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Glc8	Glc8 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Gip3	Gip3 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Gip4	Gip4 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS305-pGal1-VirD5	pGal1-His-VirD5-Ter PCR using pMVHis-VirD5 as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pGal1-VirD5NT-3xNLS	pGAL1-His-VirD5 (1-505)3xNLS-Ter PCR using pMVHis-VirD5NT as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pMET25-GFP	PCR of pMET25-GFP using pUG34GFP as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pMET25-GFP-VirD5NT	PCR of pMET25-GFP PCR using pUG34GFP as template was inserted into SpeI-SalI of pRS305.	This study
pRS306-pGAL1-VirD5	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5 as template was inserted into SpeI-SalI of pRS306.	This study
pRS306-pGAL1-VirD5NT-3xNLS	pGAL1-His-VirD5 (1-505)3xNLS-Ter PCR using pMVHis-VirD5NT as template was inserted into SpeI-SalI of pRS306.	This study

Table 4. Yeast strains used in this study.

Yeast strain	Genotype	Sources/ references
BY4743	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0)	(Brachmann *et al*., 1998)
BY4743-Δspt4	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0- Δspt4-KanMX- Δspt4-KanMX)	Eurosarf
BY4743-HTA2-CFP	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX)	(Sakalis, 2013)
BY4743-HTA2-CFP-VirD5	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX, pGAL1-His-VirD5-LEU2, pGAL1-His-VirD5-LEU2)	This study
BY4743-HTA2-CFP-GFP	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,pMET25-GFP-LEU2, pMET25-GFP-LEU2)	This study
BY4743-HTA2-CFP-GFP-VirD5NT	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,pMET25-GFP-VirD5 (1-505)-LEU2, pMET25-GFP-VirD5 (1-505)-LEU2)	This study
BY4743-HTA2-CFP (pMVHis)	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,(pMVHis-URA3))	This study
BY4743-HTA2-CFP (pMVHis-VirD5NT)	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,(pMVHis-VirD5 (1-505)-URA3))	This study
BY4743-VirD5	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0, pGAL1-His-VirD5-LEU2)	This study
BY4741	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0)	(Brachmann *et al*., 1998)
BY4741-Dam1-3xGFP	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0,Dam1-3xGFP-KanMX)	This study
BY4741-Dam1-3xGFP-VirD5	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0,Dam1-3xGFP-KanMX-pGAL1-His-VirD5-LEU2)	This study
Y716	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2[lacO256 LEU2])	(Meyer *et al*., 2013)
Y716-VirD5	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2[lacO256 LEU2]; pGAL1-His-VirD5-URA3)	This study
Y716-VirD5NT-3xNLS	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2 [lacO256 LEU2]; pGAL1-His-VirD5(1-505)3xNLS-URA3)	This study
MAS101	S. cerevisiae pRS306 [PHIS3-GFP-TUB1]	(Straight *et al*., 1997)
MAS101-VirD5	MAS101- pGAL1-His-VirD5-LEU2	This study
W303a	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15)	(Thomas and Rothstein, 1989)
W303a-Rad52-GFP	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP)	This study
W303a-Rad52-GFP-VirD5	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP,pGAL1-His-VirD5-URA3)	This study

W303a-Rad52-GFP-VirD5NT-3xNLS	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP, pGAL1-His-VirD5 (1-505)-URA3)	This study
YB3039	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ	(Norden *et al.*, 2006)
YB3039-Δspt4	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ,Δspt4	This study
YB3039(34VCN)	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ (pUG34VCn)	This study
YB3039(34VCN-VirD5)	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ (pUG34VCn-VirD5)	This study

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