ODF1 Phosphorylation by Cdk5/p35 Enhances ODF1-OIP1 Interaction

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Key Words

ODF • OIP • Cdk • Phosphorylation

Abstract

Cdk5 and p35 are integral components of the sperm tail outer dense fibers (ODFs), which contribute to the distinct morphology and function of the sperm tail. In this study, we sought to characterize and investigate the significance of Cdk5/p35 association with ODFs. We show that ODF2 interacts with Cdk5 and p35 but not with the Cdk5/p35 heterodimer. By using deletion mutants, the ODF2 binding region in p35 was mapped to residues 122 to 198. This overlaps the Cdk5 binding region in p35, explaining the inability of ODF2 to bind to the Cdk5/p35 complex. *In vitro* phosphorylation assay showed that although Cdk5/p35 does not phosphorylate ODF2, it phosphorylates ODF1. Mass spectrometry revealed that Cdk5/p35 specifically phosphorylates Ser193 in the ODF1 C-terminal region containing the Cys-X-Pro motif, the interaction site for the novel RING finger protein, ODF1 interacting protein (OIP1), a candidate E3 ubiquitin ligase, that also localizes in the sperm tail.

Cdk5 phosphorylation of ODF1 Ser193 results in enhanced ODF1-OIP1 interaction. These findings suggest that Cdk5 may be important in promoting ODF1 degradation, and potentially, the detachment and fragmentation of the sperm tail following fertilization.

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Introduction

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that was originally thought to specifically regulate neuronal functions [1, 2] but lately has been implicated in non-neuronal cell functions as well [3]. Recently, we showed the first evidence that Cdk5 and its activator, p35, are present in the testis, particularly in elongating spermatids [4], and that Cdk5/p35 activity is associated with spermiogenesis, the post-meiotic phase of spermatogenesis when spermatids differentiate into spermatozoa. In elongating spermatids, we found that Cdk5 and p35 are distinctly localized in

tails and specifically associate with the outer dense fibers (ODFs) [4].

Outer dense fibers (ODFs) are prominent cytoskeletal elements of the sperm tail that contribute to the distinctive morphological features of the sperm's motile apparatus [5]. Previous studies have indicated that ODFs function in improving the bending torque of the tail [6], and/or protecting the sperm tail against shear forces during epididymal transport and ejaculation [7]. Analysis of the polypeptide composition of ODFs showed the presence of six major and several minor polypeptides [8, 9]. The major ODF polypeptides are rich in aspartic and glutamic acids, leucine, tyrosine, cysteine, glycine, serine, and proline [8, 10], and have abundant phosphoserine residues [8]. Indeed, ODFs have been known to exist in highly phosphorylated forms [11, 12] but its significance in sperm tail function remains to be determined. In our previous study, we showed that ODF serves as a substrate of Cdk5, the first known ODF kinase [4]. *In vivo*, we found that ODF phosphorylation is reduced in sperm tails of mice lacking the Cdk5 activator, p35. However, we have yet to determine the importance of ODF phosphorylation by Cdk5.

ODF protein interactions have been proposed to play an important role in ODF assembly and consequently, the final ODF structure [13, 14]. One of such interactions is between ODF1 and ODF2 through their leucine zipper motifs [14]. However, while ODF1 can self-associate [15], ODF2 does not [14], indicating distinct interaction mechanisms among ODFs. In an effort to further examine ODF interactions in the sperm tail, the novel ODF1-interacting protein (OIP1) was identified [13]. OIP1 apparently interacts with ODF1's C-terminal Cys-X-Pro (CXP) motif. Sequence analysis of OIP1 revealed that it is a member of the RING finger family of proteins, its RING finger sequence possibly mediating its interaction with ODF1. Members of the RING finger family are often E3 ubiquitin protein ligases.

In this study, we explored the basis and consequence of the biochemical interactions among Cdk5, p35, ODFs and OIP1. We provide evidence that ODF2 and Cdk5 share a common binding region in p35 and thus, the lack of interaction between the Cdk5/p35 complex and ODF2. However, Cdk5-ODF2 and p35-ODF2 interactions seem to indicate that ODF2 serves as a scaffold for Cdk5 and p35 in an inactive state. While ODF1 does not form a stable complex with Cdk5 or p35, it serves as a substrate for Cdk5/p35. ODF1 phosphorylation by Cdk5 at Ser193 increases its interaction with OIP, possibly promoting degradation of ODF1.

Materials and Methods

Preparation of GST- and MBP-fusion proteins and in vitro translation

Bacterially expressed GST-Cdk5 and GST-p25 (the 25-kDa potent proteolytic fragment of the Cdk5 activator, p35) were purified using GSH-agarose beads following our established protocol [16]. GSH-agarose bead-bound forms of the various GST-fusion proteins containing C-terminal and N-terminal deletions of p35 were purified as described [17]. Generation of the N- and C-terminal truncated forms of p35 (residues 109-291, 122-291, 147-291, 200-291, 109-279, 109-251, 109-198, 109-179, 109-159, and 109-139) in pGEX-KG was described previously by Poon et al. [18]. ODF1, ODF2 and OIP1 were *in vitro* translated using TNT[®] rabbit reticulocyte lysate systems (Promega) in the presence of [³⁵S] methionine and cysteine [13, 14]. MBP-ODF1, MBP-ODF1 (CT) and MBP-ODF2 were expressed in bacteria and isolated using amylose-agarose beads [14]. MBP-ODF1 contains full length ODF1 fused to maltose binding protein (MBP). MBP-ODF1 (CT) contains the C-terminal half of ODF1 (residues 147-245) linked to MBP, and MBP-ODF2 contains residues 387-499 linked to MBP.

In vitro binding assays

To assess binding *in vitro*, ³⁵S-labeled ODF1 and ODF2 were incubated with GST-Cdk5, GST-p25 or reconstituted GST-Cdk5/GST-p25. Coimmunoprecipitation of ODF1 or ODF2 with Cdk5 and/or p35 by Cdk5 (C-8) or p35 (C-19) antibodies (Santa Cruz Biotech.) was detected by SDS-PAGE followed by autoradiography. On the other hand, interaction between the truncated forms of p35 and MBP-ODF2, containing a leucine zipper motif, was assessed by incubating the GSH-agarose bead-bound forms of the various p35 GST-fusion proteins containing C-terminal and N-terminal deletions with MBP-ODF2 at room temperature for 2 hours. Interaction was determined by precipitation and subsequent washing with 1x PBS containing 1 mM DTT and 0.2 % Tween-20. The precipitates were subjected to SDS-PAGE followed by immunoblot analysis using ODF2-specific antibodies [14].

Kinase assays

The ODF1 peptide phosphorylating activity of Cdk5/p35 was evaluated following methods that we used previously [16]. The reaction was initiated by the addition of 100 μ M [γ -³²P] ATP and 100 μ M ODF peptide and allowed to proceed for 2 hrs at 30°C. The amount of [γ -³²P] phosphate incorporated into the ODF1 peptide was measured using a Beckman LKB 1215 scintillation counter.

Dephosphorylation and ODF phosphorylation assay

Isolation of sperm and sperm tails, and ODF purification were performed as described previously [4]. Purified ODF was resolved by SDS-PAGE, transferred onto nitrocellulose membrane and dephosphorylated using alkaline phosphatase. GST-Cdk5 and GST-p25 were then added to kinase assay buffer [16] and phosphorylation of nitrocellulose-bound ODF was allowed to proceed for 1 hr at room temperature in the presence of 100 μ M [γ -³²P] ATP. ODF phosphorylation by Cdk5/p25 was

detected by autoradiography. To further examine ODF1 phosphorylation by Cdk5/p35, we initially mixed MBP-ODF1 (CT) (5 µg) with amylose beads (30 µl) and incubated the mixture with factor Xa to remove the MBP portion. ODF1 (CT) was then subjected to phosphorylation by Cdk/p25 in the presence of [γ - 32 P] ATP/Mg $^{2+}$ and phosphorylation was detected by autoradiography following SDS-PAGE.

Peptide sample preparation and LC-MS/MS analysis

Two ODF1 peptides containing potential Cdk5/p35 phosphorylation sites (VKIESPCYPCTSPCNPCNPCSPCPCGP and CYPCTSPCNPCNPCSPCPCGP) were incubated with GSH-agarose bead bound form of Cdk5/p25 to generate *in vitro* phosphorylated ODF1. Phosphorylated peptides were recovered by centrifugation and subsequently analyzed by LC-MS/MS on a Micromass Q-ToF-2TM mass spectrometer coupled with a Waters CapLC capillary HPLC (Waters Corp., USA) at the Institute for Biomolecular Design at the University of Alberta [19].

Immunoprecipitation, SDS-PAGE and Western blotting

Samples were precleared with protein A-agarose beads and incubated with fresh beads [pre-adsorbed with either Cdk5 (C-8) or p35 (C-19) antibody; Santa Cruz Biotech.] for 2 hrs on a rotating platform at 4°C. The immunocomplexes were washed and subjected to SDS-PAGE and immunoblotting using the indicated primary antibodies as probes. The immunoreactive protein bands were visualized using the ECL detection system (Amersham Life Sciences).

Analysis of interaction between phosphorylated ODF1 and OIP1

MBP-ODF1 (5 µg) was subjected to phosphorylation by incubating with reconstituted GSH-agarose bead-bound form of Cdk5/p35 at different periods of time (0 to 3 hrs) in the presence of ATP/Mg $^{2+}$. Phosphorylated MBP-ODF1 was recovered by centrifugation at 3,000 rpm for 10 min and phosphorylation was assessed by SDS-PAGE followed by autoradiography. Interaction between phosphorylated MBP-ODF1 (in the presence of cold ATP/Mg $^{2+}$) and *in vitro* translated 35 S-labeled OIP1 (20 µl) was determined by incubating the mixture at room temperature for 2 hours, washing, SDS-PAGE and autoradiography.

Results

Cdk5 and p25 bind to ODF2 but not ODF1

Previously, we showed that Cdk5 and p35 are integral components of ODFs in elongating spermatid tails [4]. We also found that ODFs purified from rat sperm tails consist mainly of ODF1 and ODF2. Therefore, we sought to determine whether Cdk5 and p35 bind to ODF1 and ODF2. GST-Cdk5 and/or GST-p25, a potent 25-kDa proteolytic fragment of p35, were incubated with *in vitro* translated ODF1 or ODF2 and the protein mixtures were

Fig. 1. Cdk5 and p25 bind to ODF2, but not ODF1. [35 S] Methionine and cysteine-labeled ODF2 (A) and ODF1 (B) were incubated with GST-Cdk5 (0.02 µg; lane 4), GST-p25 (0.02 µg; lane 5) or reconstituted GST-Cdk5/GST-p25 (0.02 µg/each; lanes 6 and 7). The mixtures were then subjected to immunoprecipitation using a Cdk5 or p35 antibody, and immunoblotted using ODF1 or ODF2 antibody. Lane 1 shows the equivalent of the reticulocyte lysate used in the experiment.

subjected to immunoprecipitation using a Cdk5- or p35-specific antibody. As shown in Fig. 1A (lane 1), *in vitro* translated ODF2 exhibits a relative molecular size of 84 kDa with smaller encoded products that are likely derived from translation at the internal AUG codons on the ODF2 mRNA [14]. As expected, ODF2 did not precipitate with protein A agarose beads upon addition of Cdk5 and p35 antibodies alone (lane 2) nor upon addition of Cdk5 and p25 proteins alone (lane 3). However, when ODF2 was mixed with Cdk5 (lane 4) or p25 (lane 5), followed by immunoprecipitation using the corresponding antibody (i.e.

to Cdk5 or p35), coimmunoprecipitation of ODF2 was observed, indicating that ODF2 binds to both Cdk5 and p25. By incubating ODF2 with pre-reconstituted Cdk5/p25 heterodimer followed by immunoprecipitation using a Cdk5 (lane 6) or p35 (lane 7) antibody, no detectable ODF2 coimmunoprecipitation was observed. Thus, while ODF2 binds to Cdk5 and p35, it does not interact with the Cdk5/p35 heterodimer. Conversely, ODF1 did not show any significant interaction with Cdk5, p25 or the Cdk5/p25 heterodimer (Fig. 1B).

The ODF2 binding region in p25 overlaps the Cdk5 binding region and resembles a leucine zipper motif

ODF2 contains a functional leucine zipper motif in its C-terminal region through which it interacts with other proteins including ODF1 [14]. To determine whether an ODF2 fragment (MBP-ODF2; residues 387-499) containing the leucine zipper sequence [14] binds to p25, MBP-ODF2 was incubated with GST-p25 (designated as aa109-291), and interaction between the two proteins was detected by precipitation followed by immunoblot analysis using an antibody to ODF2. The presence of immunoreactive bands in Fig. 2A, lane 2, indicated interaction between p25 and MBP-ODF2 with p25 potentially binding to the leucine zipper motif in ODF2. We then sought to define the region in p25 that is required for ODF2 binding. To do so, various N- and C-terminally truncated forms of p25 were expressed in *E. coli*, and purified GSH-agarose bead-bound forms of these peptides were used to test for their ability to precipitate the leucine zipper-containing MBP-ODF2. Schematic representations of p35 and its deletion mutants as well as their interactions with MBP-ODF2 are shown in Fig. 2B, with the corresponding precipitate immunoblot data shown in Fig. 2A. Deletion of the p35 N terminus up to residue 121 (aa122-291) did not affect its ability to bind ODF2. However, deletion up to residue 146 (aa147-291) abolished binding to ODF2. On the other hand, deletion of the p35 C-terminus down to residue 197 (aa109-198) did not inhibit binding to ODF2 but further deletions (aa109-179, aa109-159, aa109-139) resulted in a complete loss of binding to ODF2. These data indicate that the ODF2 binding region in p35 lies in residues 122 to 198. This region overlaps the Cdk5 binding region in p35 (Fig. 2B) previously identified by Poon et al. (residues 150-200) [18] and Tang et al. (residues 150-291) [20], explaining the inability of ODF2 to bind to the Cdk5/p35 complex. It is interesting that the ODF2 binding region in p35 (residues 122-198) contains a stretch of 3 heptade repeats of leucine residues

Fig. 2. ODF2 binds to p35 residues 122-198. Interaction of the various truncated forms of p35 with ODF2 was determined as described in Experimental Procedures. (A) shows the resulting blot of the interaction assay and (B) shows a schematic representation of the truncated forms of p35 as well as the ODF2 (based on this study) and Cdk5 [18, 20] binding region in p35. Note that residues 109-291 represent p25.

(Fig. 3), resembling a leucine zipper motif. This is consistent with the finding that the p35 region contains two of the α -helices previously observed by crystal structure analysis [20, 21].

Cdk5 phosphorylates Ser193 in ODF1

ODF polypeptides are generally rich in serine and proline [8, 10] and have abundant phosphoserine residues [8]. Sequence analysis shows that the C-terminus of ODF1 contains four Ser-Pro residues (Fig. 4A) that are potential phosphorylation sites for Cdk5, a proline-directed serine/threonine protein kinase. Indeed, incubation of purified rat sperm tail ODF with Cdk5/p25 in the presence of [γ - 32 P] ATP/Mg $^{2+}$ revealed phosphorylation of ODF1 (Fig. 4B, lane 2), corresponding to the 32- and 26-kDa

				<u>αNT</u>	
122	sssvkkaph	pavtsagtpk	rvivqastse	lLrclgefLc	rrcyrLkhls 170
	<u>α1</u>				
171	ptdpvlwlrs	vdrslllqgw	qdqgfitp		198

A

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1 maalscllds vrrdikkvdr elrqlrcide issrclcdly mhpycccdlh 50
51 pypyclcysk rsrscglcdl yypcclcdyk lycrlrpslrs lerlrtrtnr 100
101 ilassccssn ilgsvnvcgf epdqvkvrvk dgkvcvsaer enrydcclgsk 150
151 kysymnicke fslppcvdek dvtysyglgs cvkieSPcyp ctSPcnpncnp 200
201 cSPcSPcpgpc gpcgpcgpcg pcgpcdpcnp cypcgsrfsc rkmil 245
```

B

75 -
50 -
35 -
25 -
kDa

1 2 3

84
80
71
56
40] ODF2

32
26] ODF1

ODF Prep + + +
Cdk5/p25 - + -
[γ - 32 P] ATP/Mg $^{2+}$ + + -

C

21 -
kDa

1 2

ODF1(CT) + +
Cdk5/p25 - +
[γ - 32 P] ATP/Mg $^{2+}$ + +

D

ODF1 peptide phosphorylation (cpm $\times 10^{-4}$)

n=3

1.0
0.5
0

1 2 3 4

E

Residues	Sequence	Theoretical m/z	Observed m/z
182-187	VKIESP	654.4	655.2
182-192	VKIESPCYPCT	1221.6	1220.3
188-195	CYPCTSPC + one phosphorylation	935.3	934.7
188-195	CYPCTSPC	855.3	-
199-209	NPCSPCSPCGP	1061.5	1062.8

these observations, we found that ODF1 (CT), containing the ODF1 C-terminal region (residues 147-245), can be phosphorylated by Cdk5/p25 (Fig. 4C). Likewise, an

Fig. 5. ODF1 Ser193 phosphorylation by Cdk5/p25 results in enhanced ODF1-OIP1 interaction. MBP-ODF1 phosphorylation by Cdk5/p25 was performed as described in Materials and Methods. Lanes 1, 2, 3 and 4 represent incubation times of 0, 0.5, 1, and 3 hrs, respectively. Considerable phosphorylation of ODF1 was observed following a 3-hr incubation with Cdk5/p25 (lane 4, upper panel). Phosphorylated ODF1 shows enhanced interaction with OIP1 (corresponding lane 4, lower panel).

Fig. 6. Helical wheel model of the amphipathic nature of p35 α NT, containing a stretch of 3 heptade repeats of leucine residues.

ODF1 peptide containing the four potential Cdk5 phosphorylation residues (VKIES₁₈₆PCY-PCTS₁₉₃PCNPCNPCS₂₀₂PCS₂₀₅PCGP) was phosphorylated by Cdk5/p25 (Fig. 4D). We then sought to identify the Cdk5 phosphorylation site in ODF1 by mass spectrometry. Analysis of a tryptic digest of MBP-ODF1 proved to be inappropriate as the tryptic peptide fragment containing the potential Cdk5 phosphorylation residues was too long (54mer, residues 184-237), making fragmentation analysis difficult. Therefore, we have generated two peptides (VKIES₁₈₆PCY-PCTS₁₉₃PCNPCNPCS₂₀₂PCS₂₀₅PCGP and CYPCTS₁₉₃PCNPCNPCS₂₀₂PCS₂₀₅PCGP) containing the potential Cdk5 phosphorylation sites. Following *in vitro* phosphorylation of these peptides by Cdk5/p25 in the presence of ATP/Mg²⁺, a combination of LC-MS/MS analysis was performed to identify the Cdk5 phosphorylation site. Our MS data showed that the fragmented peptide containing Ser193 (C₁₈₈YPCTS₁₉₃PC₁₉₅) was the only fragment that showed evidence of phosphorylation (Fig. 4E). No phosphorylation signal was detected in the other peptide fragments that

also contain Ser-Pro sites. Thus, our data indicate that Ser193 is the phosphorylation target of Cdk5 in ODF1.

Cdk5/p35 phosphorylation of ODF1 Ser193 results in enhanced ODF1-OIP1 interaction

The Cdk5 phosphorylation site in ODF1, Ser193, is located in the evolutionally conserved Cys-X-Pro repeats (residues 187 to 233) in the C-terminus. By yeast two hybrid analysis, this motif was identified as the specific binding region for the RING finger protein, ODF1-interacting protein 1 (OIP1) [13]. This is interesting in that OIP1 was shown to be mostly expressed during spermiogenesis [13], overlapping the expression of Cdk5 and p35 [4]. To examine the potential significance of ODF1 phosphorylation by Cdk5/p35, we investigated the possibility that phosphorylation of ODF1 Ser193 by Cdk5 affects ODF1 interaction with OIP1. Indeed, as shown in Fig. 5, ODF1 phosphorylated by Cdk5/p35 over three hours (upper panel, lane 4) exhibited a four-fold increase in association with OIP1 (lower panel, lane 4), indicating that Cdk5 phosphorylation of ODF1 results in enhanced ODF1-OIP1 interaction.

Discussion

There is now considerable evidence that Cdk5 plays a role in postmitotic functions of non-neuronal cells. For example, we [22] and others [23, 24] have shown that Cdk5 is involved in the signaling pathway that leads to muscle differentiation. Cdk5 is also involved in secretion by pancreatic β -cells [25, 26] and neutrophils [27]. Our recent studies suggest that Cdk5 is further involved in spermiogenesis [4]. To investigate the potential role of Cdk5 in the sperm, we examined the basis and consequence of our earlier observation that Cdk5 and p35 strongly associate with sperm tail ODFs [4].

We recently observed that ODF1 and ODF2 are major components of ODFs purified from rat sperm tails [4] and here, we show that each of these ODFs exhibits unique interactions towards Cdk5/p35. ODF2 forms a stable complex with Cdk5 and p35 but does not interact with the Cdk5/p35 heterodimer. The overlapping ODF2 and Cdk5 interaction site in p35 explains the lack of ODF2 interaction with the Cdk5/p35 complex. Furthermore, the absence of ODF2-Cdk5/p35 interaction suggests that the ODF2 binding region in Cdk5 becomes inaccessible when Cdk5 forms a complex with p35 or overlaps the p35 binding site. Further investigation is required to test this premise.

Previously, it was shown that leucine zippers mediate the association of ODF2 with ODF1 [14]. Here, we found that p35 associates with an ODF2 fragment containing a leucine zipper motif. It is interesting that the p35 region that interacts with ODF2 also contains leucine residues in a stretch of 3 heptade repeats reminiscent of a leucine zipper motif. Incidentally, a portion of this stretch of residues forms the first α -helix, designated as N-terminal α -helix or α NT, in p35 [21]. Consistent with leucine zipper motifs that play a critical role in protein-protein and self interaction, the p35 α NT may form an amphipathic α -helix (Fig. 6). Indeed, α NT forms a helix-loop-helix structure with α 1 in p35 [21]. The premise that the α NT region in p35 may be important for ODF2 binding is consistent with the finding that Cdk5 interacts with a peptide corresponding to the p25 α NT helix [28] as well as reports that the Cdk5 binding region in p35 includes the α NT residues as deletion of the latter in p35 results in loss of binding with Cdk5 [18, 20]. These studies are further compatible with the lack of ODF2 interaction with the Cdk5/p35 complex due to common ODF2 and Cdk5 binding residues in p35. Nonetheless, the stable ODF2-Cdk5 and ODF2-p35 interactions led to our speculation

that ODF2 serves as a protein scaffold for Cdk5 and p35 in an inactive state.

In accord with our finding that ODF2 does not interact with the Cdk5/p35 complex, ODF2 does not serve as a substrate of Cdk5/p35. On the other hand, ODF1, which does not form a stable physical complex with Cdk5/p35, acts as a substrate of Cdk5/p35. This is not surprising as ODF1 contains four potential Cdk5 phosphorylation sites (Ser-Pro residues) in its C-terminus. Indeed, Cdk5 phosphorylation of ODF1 from rat sperm tail as well as bacterially expressed ODF1 (CT) and an ODF1 peptide containing its four potential phosphorylation residues indicate that ODF1 is a substrate of Cdk5. While the precise functions of ODF1 and ODF2 are still unknown, our premise is that both ODF2-Cdk5 and ODF2-p35 interactions, and ODF1 phosphorylation at Ser193 by Cdk5/p35 serve important functions in the sperm. Perhaps during fertilization, the ODF2-associated inactive Cdk5 and p35 pools are released and form active Cdk5/p35 complexes that phosphorylate their substrates, including ODF1.

The Cdk/p35 target in ODF1, Ser193, lies in the C-terminus Cys-X-Pro repeats which have been identified as the binding region for OIP1 [13]. As with Cdk5 and p35 [4], OIP1 [13] is expressed during spermiogenesis. Interestingly, ODF1 Ser193 phosphorylation by Cdk5 enhances ODF1 interaction with OIP1. As a RING finger protein, OIP1 is a candidate E3 ubiquitin ligase that together with an E1 ubiquitin-activating enzyme and an E2 ubiquitin-conjugating enzyme, marks proteins for degradation by the proteasome [13]. It is possible that OIP1 is part of a multi-protein E3 complex in the sperm that participates in the degradation of ODF components. Maybe, upon fertilization, sperm tail OIP1 is released from its protein scaffold and Cdk5/p35 phosphorylates ODF1 Ser193. These events allow increased ODF1-OIP1 interaction and may promote ODF1 degradation, contributing to the fragmentation and detachment of the sperm tail following fertilization.

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