Par-1 and Tau regulate the anterior–posterior gradient of microtubules in *Drosophila* oocytes

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**Abstract**

The formation of an anterior–posterior (AP) gradient of microtubules in *Drosophila* oocytes is essential for specification of the AP axis. Proper microtubule organization in the oocyte requires the function of serine/threonine kinase Par-1. The N1S isoform of Par-1 is enriched at the posterior cortex of the oocyte from stage 7 of oogenesis. Here we report that posterior restriction of Par-1 (N1S) kinase activity is critical for microtubule AP gradient formation. Egg chambers with excessive and ectopic Par-1 (N1S) kinase activity in the germline cells display phenotypes similar to those of egg chambers treated with the microtubule-depolymerizing drug colcemid: depolymerization of microtubules in the oocyte and disruption of oocyte nucleus localization. A phosphorylation target of Par-1, the microtubule-associated protein Tau, is also involved in oocyte polarity formation, and overexpression of Tau alleviates the phenotypes caused by ectopic Par-1 (N1S) kinase activity, suggesting that Par-1 regulates oocyte polarity at least partly through Tau. Our findings reveal that maintaining proper levels of Par-1 at correct position in the oocyte is key to oocyte polarity formation and that the conserved role of Par-1 and Tau is crucial for the establishment of an AP gradient of microtubules and for AP axis specification.

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**Introduction**

Localization of mRNAs that specify the anterior–posterior (AP) axis in *Drosophila* oocytes depends on the polarity of the microtubules. In the oocyte, the microtubule nucleating activity is associated with the centrosome–nucleus complex (Januschke et al., 2006), which is located at the posterior end of the oocyte during stages 1 to 6 of oogenesis. After stage 6, microtubule reorganization occurs when the oocyte receives an unknown signal from the posterior follicle cells and the oocyte nucleus migrates anteriorly (Januschke et al., 2006). Reorganization of the microtubule organizing center allows formation of a gradient of microtubules from higher density at the anterior to lower density at the posterior (Dollar et al., 2002; Januschke et al., 2006; Theurkauf et al., 1992). On the basis of the new microtubule polarity, maternal determinants such as bicoid and oskar mRNAs are localized at the anterior and posterior, respectively, whereas gerken (grk) associates with the oocyte nucleus in the anterior dorsal corner (Riechmann and Ephrussi, 2001).

The conserved serine/threonine protein kinase Par-1 has been shown to play important roles in cellular asymmetry and polarization in a variety of systems (Bohm et al., 1997; Guo and Kemphues, 1995; Shulman et al., 2000; Tomancak et al., 2000). In *Caenorhabditis elegans*, PAR-1 protein is required for AP axis formation (Guo and Kemphues, 1995). *Drosophila* Par-1 is also required for AP polarization of the oocyte at two stages of oogenesis. In germline clones of a par-1 null allele, the oocyte fails to polarize during early oogenesis and reverts to a nurse-cell fate, producing egg chambers with 16 nurse cells and no oocyte (Cox et al., 2001; Huynh et al., 2001). Analysis of hypomorphic par-1 alleles that allow oocyte development past early oogenesis revealed that par-1 is required for repolarization of the oocyte microtubule cytoskeleton (Shulman et al., 2000; Tomancak et al., 2000). Our recent study showed that *igl* regulates oocyte polarity and microtubule polarity by regulating the Par-1 posterior enrichment (Tian and Deng, 2008). The par-1 gene encodes multiple isoforms, including N1S, N1L, N2S and N3L. Previous studies showed that only Par-1 (N1S) and Par-1 (N1L) can rescue the oocyte polarity defect phenotypes in par-1 mutant (Doerflinger et al., 2006), but how Par-1 (N1S) or (N1L) regulates the microtubule polarity in the oocyte remains unclear.

In mammals, the Par-1 homologues MARK1 and MARK2 phosphorylate microtubule-associated proteins (MAPs), such as Tau, MAP2, and MAP4, in a conserved KXGS motif (Drewes et al., 1997; Ebnet et al., 1999; Illebenberger et al., 1996). This phosphorylation inhibits the binding of MAPs to microtubules, results in instability of microtubules (Ebnet et al., 1999). Overexpression of MARKs in cells could lead to hyperphosphorylation of these MAPs on the KXGS motif and cause disruption and disappearance of microtubules. In *Drosophila*, Tau is phosphorylated by Par-1 in vitro and in vivo, and this phosphorylation is a prerequisite to a series of phosphorylation processes that generate toxic Tau in neurons (Nishimura et al., 2004).
In the oocyte, the relationship between Par-1 and Tau is unclear. Although Tau has a clear AP gradient, similar to that of the microtubules in the oocyte (Shulman et al., 2000), the microtubule organization defect has not been detected in tau germline clones before. Here, we show that Tau is involved in the regulation of oocyte microtubule polarity and that the establishment of the AP gradient of microtubules depends on Par-1. Excessive kinase activity of Par-1 disrupts oocyte polarity, causing defects that resemble those caused by the microtubule-depolymerizing drug colcemid. The oocyte polarity and microtubule defects in egg chambers with ectopic Par-1 kinase activity are alleviated when Tau is cooverexpressed, suggesting that Tau is a target of Par-1 in the oocyte to regulate the microtubule polarity. Our studies reveal the importance of localized and appropriate levels of the Par-1 kinase activity in the establishment of oocyte polarity, and the conserved role of Par-1 and Tau in the organization of microtubules in the oocyte.

Materials and methods

Fly strains and genetics

The strains were raised at 25 °C on standard media. We used Df(3R) MR22(tau) as a null allele for tau (Doerflinger et al., 2003; tau germline clones were generated by FLPe-RRT-induced mitotic recombination (Xu and Rubin, 1993) from the following strains: FRT82B-Df (3R)MR22(tau)/TM3, Ser act:GFP and w; hve', mat-tub-alpha4:GFP Stauwen), fts'; hs:FLP; P(neoFRT82B P(Ubi-GFP)83/TM3, Sb (Martin et al., 2003). We induced germline clones by administering 2-h 37 °C heat shocks on two consecutive days during the third instar. For Par-1 (N1S) and Tau overexpressions, we obtained transgenic fly stocks with pUASP: GFP-Par-1 (N1S) on the second chromosome, pUASP: GFP-Par-1 (N1S) with mutant AEM (AEM*) and pUASP: GFP-tau from D. St Johnston (Doerflinger et al., 2003; Doerflinger et al., 2006; Huynh et al., 2001) and transgenic fly stocks with pUASP: GFP-Par-1 (N1S) ΔSP and pUASP: GFP-Par-1 (N1S) ΔK* on the third chromosome from A. Ephrussi (Vaccari et al., 2005). Mata4-tubulin-Gal4-VP16 (Mat-Gal4) was used for the overexpression in the germline.

Molecular biology and biochemical analysis

To generate transgenic fly lines carrying the Par-1 (N1S) kinase domain (Par-1 (N1S) ΔSP), we amplified the par-1 (N1S) CDNA (a gift from A. Ephrussi) (Vaccari et al., 2005) using the primers (5'-GGATCCATCTGGACGCAATGC-3' and 5'-TCTAGATTCTAAACCCGTT-3'), but excluding the spacer domain that is required for Par-1 (N1S) localization. The PCR product was subcloned into the pUASP vector (North, 1998) and sequenced. Transgenic lines of the pUASP:Par-1(N1S)ΔSP construct were generated by standard methods and overexpressed by means of the germline Gal4 drivers (Mat-Gal4). Two independent transgenic lines were used for phenotypic analysis in this study.

Antibody staining, imaging, and analysis

Antibody stainings were performed according to standard procedures. Primary antibodies were diluted as follows: rabbit anti-Stau, 1:5000 (St Johnston et al., 1991); mouse anti-Gurken, 1:20 (Developmental Studies Hybridoma Bank (DHSB)). For microtubule staining, samples were fixed for 10 min in 8% paraformaldehyde (Doerflinger et al., 2003) and stained with a FITC-conjugated monoclonal anti-α-Tubulin antibody DM1A (1:400; Sigma). Secondary antibodies conjugated to Alexa Fluor 546 goat anti-mouse and goat anti-rabbit (Molecular Probes) were used at 1:400. Fluorescently labeled samples were counterstained with DAPI for visualization of DNA. Images were captured with a Zeiss LSM 510 confocal microscope and assembled in Adobe Photoshop.

Colcemid treatments of the flies

For the colcemid treatment, flies were fed yeast paste containing 100 μg ml⁻¹ colcemid for 18 h.

Results

Ectopic Par-1 kinase causes Gurken/oocyte nucleus mislocalization

Posterior Par-1 enrichment in the oocyte cortex is visualized through expression of one copy of the GFP-tagged form of Par-1 (N1S) (GFP-Par-1 (N1S)) in the germline (Doerflinger et al., 2006; Tian and Deng, 2008) (Figs. 1C, G'). In these egg chambers, the oocyte polarity markers, Grk/oocyte nucleus (Figs. 1C, C') and Stau (figs. colocalizes with the oskar mRNA at the posterior) (Figs. 1D, D'), were localized during stages 9–10 at their expected anterior-dorsal corner and posterior positions, respectively (Figs. 1A, B). To determine whether egg chambers can tolerate increased amounts of Par-1 (N1S), we overexpressed two copies of UASp-GFP-Par-1 (N1S) using the Mat-Gal4 driver in the oocyte. The oocytes showed increased localization of GFP-Par-1 (N1S) at the cortex and in the cytoplasm, in addition to its posterior enrichment (Figs. 1H, H'), suggesting that Par-1 is still enriched at the posterior when an excessive amount of Par-1 is present in the oocyte. These egg chambers however displayed severe defects in the localization of Grk/oocyte nucleus and Stau during stages 9–10: the oocyte nucleus was frequently found in the center of the oocyte, and Grk was diffusely localized in the cytoplasm of the oocyte (23%, n = 110; Figs. 1E, F'), and Stau showed reduced localization at the posterior and an expansion toward the middle portion of the oocyte cortex (14%, n = 123; Figs. 1E, F'). To determine whether the Grk/oocyte nucleus localization defect is caused by increased Par-1 kinase activity at the anterior and/or in the cytoplasm of these egg chambers, we asked whether generation of ectopic and mislocalized Par-1 (N1S) kinase activity in the oocyte through other means would also disrupt the oocyte polarity. Two different approaches were used to generate ectopic Par-1 kinase activity in the oocyte. First, we overexpressed a mutant form of Par-1 (N1S) with a mutated antero-lateral exclusion motif (AEM*), which is required for Par-1 (N1S) posterior enrichment in the oocyte (Doerflinger et al., 2006). Second, we produced mislocalization of Par-1 (N1S) by co-overexpression of Lgl-3A and GFP-Par-1 (N1S) in the oocyte which we have previously shown to lead to mislocalization of Par-1 (N1S) from the posterior (Tian and Deng, 2008). Thus, in these experiments we generated egg chambers that had both excessive and mislocalized Par-1 (N1S), note that only one copy of Par-1 (N1S) was overexpressed in these egg chambers (Fig. 2). When Par-1 (N1S) AEM* was overexpressed in the germ line, Par-1 (N1S) was localized along the entire cortex and in the cytoplasm (Fig. 2F) (Doerflinger et al., 2006). Consistent with a role for ectopic Par-1 activity in anterior/cytoplasmic locations in disruption of oocyte polarity, the oocyte nucleus was localized in the center of the oocyte (97%, n = 152; Fig. 2D, arrow) (Doerflinger et al., 2006) and Stau was weakly localized along the cortex (90%, n = 111; Fig. 2E). In egg chambers with overexpression of Par-1 (N1S) and Lgl-3A (Figs. 2G and H) the phenotypes were similar to that of egg chambers with two copies of GFP-Par-1 (N1S) overexpression (Figs. 1E, F). Specifically, the oocyte nucleus was frequently mislocalized to the center of the oocyte (43%, n = 141; Fig. 2G) and the posterior Stau was reduced and localized along the lateral cortex (35%, n = 123; Fig. 2H). This phenotype differs from expression of either single construct: Par-1 (N1S) alone shows no defects (Figs. 1C, D), while Lgl-3A alone causes the oocyte nucleus/Grk to be mislocalized to the oocyte posterior and Stau to the center of the oocyte (Tian and Deng, 2008) (Figs. 2J, K). These experiments indicate that ectopic Par-1 (N1S) activity in non-posterior regions of the oocyte can disrupt oocyte polarity.

In the oocyte, the relationship between Par-1 and Tau is unclear. Although Tau has a clear AP gradient, similar to that of the microtubules in the oocyte (Shulman et al., 2000), the microtubule organization defect has not been detected in tau germline clones before. Here, we show that Tau is involved in the regulation of oocyte microtubule polarity and that the establishment of the AP gradient of microtubules depends on Par-1. Excessive kinase activity of Par-1 disrupts oocyte polarity, causing defects that resemble those caused by the microtubule-depolymerizing drug colcemid. The oocyte polarity and microtubule defects in egg chambers with ectopic Par-1 kinase activity are alleviated when Tau is cooverexpressed, suggesting that Tau is a target of Par-1 in the oocyte to regulate the microtubule polarity. Our studies reveal the importance of localized and appropriate levels of the Par-1 kinase activity in the establishment of oocyte polarity, and the conserved role of Par-1 and Tau in the organization of microtubules in the oocyte.
To further test the relationship between ectopic Par-1 kinase activity and Grk/oocyte nucleus localization, we examined oocyte polarity markers in egg chambers with germline overexpression of either a kinase-dead version of Par-1 (N1S) \textit{(GFP-Par-1 (N1S) K*)} or a truncated form of Par-1 (N1S) that contains the kinase domain but lacks the spacer domain necessary for Par-1 localization \textit{(GFP-Par-1 (N1S) ΔSP)} \cite{Vaccari et al., 2005}. Par-1 (N1S) K* showed a localization similar to that of the wild-type Par-1 (N1S) (Figs. 2N and Q), but no defect in Grk/oocyte nucleus or Stau localization was observed in egg chambers with one or two copies of Par-1 (N1S) K* overexpression (Figs. 2L, M, O, and P). In addition, oocytes with cooverexpression of Lgl-3A and Par-1 (N1S) K* showed a localization similar to that of the wild-type Par-1 (N1S) (Figs. 2N and Q), but no defect in Grk/oocyte nucleus or Stau localization was observed in egg chambers with one or two copies of Par-1 (N1S) K* overexpression (Figs. 2L, M, O, and P). In addition, oocytes with cooverexpression of Lgl-3A and Par-1 (N1S) K* displayed phenotypes similar to that of Lgl-3A overexpression alone (data not shown), but did not have defects similar to egg chambers with cooverexpression of Lgl-3A and the wild-type Par-1 (N1S), as described above. In contrast, overexpression of Par-1 (N1S) ΔSP, which was uniformly localized throughout the cortex and cytoplasm of the oocyte (Figs. 3G, G'), caused severe defects in Grk/oocyte nucleus localization. The oocyte nucleus was almost always found in the center of these oocytes with diffuse Grk (96%, \(n = 111\); Figs. 3C, C'), as well as weak Stau staining at the posterior and the middle portion of the oocyte cortex (87%, \(n = 103\); Figs. 3D, D'). Because the same \textit{Mat-Gal4} driver was used, the expression levels of different Par-1 constructs were similar (Fig. S1). Together these findings indicate that the kinase activity of ectopically localized Par-1 is essential for its effects on oocyte polarity, consistent with the known importance of the kinase function of endogenous Par-1 in the regulation of oocyte polarity \cite{Shulman et al., 2000; Tomancak et al., 2000}.

\textbf{Par-1 (N1S) kinase activity depolymerizes microtubules in the oocyte}

The mislocalization of oocyte nucleus and diffuse Grk in the cytoplasm that we observed when Par-1 (N1S) was active in non-posterior regions of the oocyte differs from that of a number of other oocyte polarity mutants, such as \textit{grk} and \textit{top} in which the oocyte nucleus remains at the posterior of the oocyte \cite{Gonzalez-Reyes et al., 1995; Roth et al., 1995}, but instead resembles the defects described in flies fed the microtubule-depolymerizing drug colcemid \cite{Cha et al., 2002}. After colcemid treatment for 18 h, the majority of oocyte nuclei at stages 9–10 were in the center of the oocyte, whereas Grk was detected at a low level in the cytoplasm of the oocyte (Figs. 3E, E'). Stau, normally localized as a crescent at the posterior, was weakly distributed along the posterior and lateral cortex in the oocyte (Figs. 3F, F'). The phenotypic similarities between egg chambers with excessive and mislocalized Par-1 (N1S) kinase activity and egg chambers with depolymerized microtubules suggest that the ectopic kinase activity of Par-1 (N1S) disrupts the microtubules in the oocyte.

To analyze microtubule organization in egg chambers with excessive and mislocalized Par-1 kinase activity, we set out to visualize microtubule distribution directly in these egg chambers. Because the microtubule organization can be clearly observed in the oocyte with a FITC-conjugated anti-\(\alpha\)-Tubulin antibody, we generated transgenic lines carrying the Par-1 (N1S) ΔSP transgene without the GFP tag. Using \textit{Mat-Gal4} to drive Par-1 (N1S) ΔSP overexpression, we found no difference in the phenotypes between these transgenes and the above-mentioned transgene with the GFP tag: oocyte nucleus mislocalization in the center of the oocyte, diffusion of Grk, and weak localization of Stau at the posterior and lateral cortex (data not shown).
shown). Stained with a FITC–anti-α-Tubulin antibody, the AP gradient of microtubules in the germline that was clearly seen in the wild type at stages 8 and 9 (Figs. 4A, B) was lost. Instead, microtubules in the germlines showed weak uniform staining throughout the oocyte (Figs. 4C, D), but the microtubules in the follicles were similar to those in the wild type because the Gal4 driver was only expressed in the germline. This pattern in the germline is similar to that in oocytes treated with colcemid for 18 h (Figs. 4E, F), indicating that excessive and mislocalized kinase activity of Par-1 (N1S) depolymerizes microtubules in the oocyte.

Involvement of Tau in the germline for oocyte polarity

The mammalian homologs of Par-1 (the MARKs) phosphorylate the repeated motif in the microtubule-binding proteins Tau, Map2, and Map4 (Drewes et al., 1995; Illenberger et al., 1996).
Phosphorylation at these sites reduces the binding affinity of these MAPs for microtubules and induces microtubule depolymerization (Drewes et al., 1997; Illenberger et al., 1996). Tau has also been shown to be phosphorylated by Par-1 in vivo and in vitro in Drosophila (Nishimura et al., 2004), but previous studies indicated no defects in tau germline clones (Doerflinger et al., 2003). To verify this result,
we reexamined the oocyte polarity in germline clones of a tau null allele, tau(Df(3R)MR22). We found that Grk and the oocyte nuclei were mislocalized to central or lateral region of the oocyte at stage 10 (58%, n = 157; Fig. 5B), but Stau remained weakly at the posterior (Fig. 5B).

This phenotype is similar to that of excessive Par-1 (N1S) overexpression or colcemid-treated egg chambers (Figs. 3C–F). To verify whether these defects were indeed caused by the tau mutation, we performed a rescue experiment and found that overexpression of Tau in tau(Df(3R)MR22) germline clones fully alleviated the oocyte-nucleus mislocalization defect (100%, n = 52; Figs. 5D–D'). These data indicate that Tau is very probably involved in maintaining microtubule stability in the oocyte.

**Overexpression of Tau alleviates the defects caused by ectopic Par-1 activity**

To explore the relationship between Par-1 (N1S) and Tau in regulating the microtubules in the oocyte, we reexamined the oocyte polarity in germline clones of a tau null allele. Overexpression of Tau-GFP (Doerflinger et al., 2003) with GFP-Par-1 (N1S) ΔSP in the germ line. These egg chambers showed reduced tau mutation, we performed a rescue experiment and found that overexpression of Tau in tau(Df(3R)MR22) germline clones fully alleviated the oocyte-nucleus mislocalization defect (100%, n = 52; Figs. 5D–D'). These data indicate that Tau is very probably involved in maintaining microtubule stability in the oocyte.

**Discussion**

The reorganization of the microtubule cytoskeleton during mid-oogenesis that leads to the formation of an AP gradient of microtubule density in the oocyte is essential for body-axis formation in Drosophila (Dollar et al., 2002; Januschke et al., 2006; Theurkauf et al., 1992), but our understanding of the mechanisms regulating this reorientation is limited. We find that restriction of Par-1(N1S) kinase activity to the posterior end of the oocyte is critical for establishment of the microtubule polarity. Par-1 function is likely mediated by phosphorylating Tau in the posterior, which destabilizes the microtubule resulting in reorientation of the microtubules and establishment of the AP gradient of microtubule density in the oocyte visible after stage 7.

Feeding female flies the microtubule-depolymerizing drug colcemid causes a severe defect in oocyte polarity: mislocalization of the oocyte nucleus in the center of the oocyte. This phenotype can be reproduced when egg chambers have overexpressed Par-1 (N1S) or Par-1 (N1S) kinase domain in the anterior and cytoplasm of the oocyte. This phenotype reflects the severe disruption of microtubule polymerization in the oocyte, which differs from the previously reported oocyte polarity mutants such as mutants in the Grk/EGFR pathway, Notch, and Hippo pathway mutants (Gonzalez-Reyes et al., 1995; Meignin et al., 2007; Polesello and Tapon, 2007; Roth et al., 1995; Ruohola et al., 1991; Yu et al., 2008). In those mutants, the posterior microtubule organizing centers (MTOCs) fail to be disrupted, therefore the oocyte likely has two ends with higher density of microtubules. The mutants with ectopic Par-1(N1S) kinase activity represent the opposite of this phenotype, no areas in the oocyte show high density of microtubules. This phenotype is also in line with the role of Par-1(N1S) in depolymerizing microtubules through Tau. It is expected that reduced or loss of Par-1 activity would result in failure to destabilize the posterior MTOCs in the oocyte. Indeed, hypomorphic alleles of Par-1, showed a phenotype that is more similar to the EGFR pathway mutants in terms of the polarity defect in the oocyte (Roth et al., 1995).

The oocyte microtubule polarity defect was not previously observed in tau germline clones (Doerflinger et al., 2003). The possible reasons are that not all tau germline clones possess oocyte polarity defects and that using α-Tubulin staining to detect the microtubule defects is not convenient when the phenotypes can be best seen at stage 10. In tau germline clones, the oocyte nuclei mislocalization was only obvious at stage 10 or later stages, and the posterior Stau staining was slightly stronger than that in oocytes with excessive Par-
1 (N1S) activity or cocemid-treated flies. Perhaps other Par-1 (N1S) targets are involved in regulating the microtubules in the oocyte.

We propose that the AP gradient of microtubules depends at least in part on an AP gradient of Tau, which is regulated by Par-1. In the wild-type oocyte, the posterior has enriched Par-1 that disassembles the microtubules by phosphorylating Tau. In egg chambers that have mislocalized and excessive amounts of Par-1 (N1S), the anterior and cytoplasmic Tau is also phosphorylated and thus disassembles the microtubules in the anterior and cytoplasm of the oocyte as well. Expressing different parts of Par-1 (N1S) in the germ line revealed the functional significance of different regions of Par-1 (N1S) protein in oocyte polarity. The posterior enrichment requires the spacer domain, whereas the kinase domain alone is sufficient to phosphorylate Tau but not enough for posterior enrichment. Considering that Lgl phosphorylation by aPKC can regulate Par-1 (Tian and Deng, 2008), our current findings suggest a model in which the AP gradient of the Tau is regulated by Par-1 kinase activity in the posterior, which is regulated by Lgl. When egg chambers have Lgl-3A (the nonphosphorylatable, active form of Lgl (Betschinger et al., 2003; Tian and Deng, 2008)) overexpression alone, we believe wild-type Par-1 is also mislocalized and therefore present at a reduced level along the oocyte cortex. Par-1 at this low level is unable to depolymerize microtubules at the posterior, at the anterior, or in the cytoplasm of the oocyte. Therefore, the microtubule organizing center is retained at the posterior and Kin;ß-Gal is mislocalized to the center of the oocyte in Lgl-3A-overexpressing egg chambers (Tian and Deng, 2008). When Lgl and Par-1 are cooverexpressed, the excessive and mislocalized Par-1 is sufficient to depolymerize microtubules throughout the oocyte cortex, thereby producing a phenotype similar to that produced by the treatment with colcemid. The Par-1 human homolog MARK is known to be able to phosphorylate Tau to regulate microtubule dynamics. Our data here, with those from our previous study (Tian and Deng, 2008), reveal that the conserved role of Par-1 in regulating microtubule polarity is essential for oocyte polarity, and the pathway of aPKC, Lgl, Par-1, and Tau is probably evolutionarily conserved and implicated in other cell types.

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Appendix A. Supplementary data


References


