Biochemical and Biophysical Research Communications 435 (2013) 610–615

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Aurora A kinase negatively regulates Rho-kinase by phosphorylation *in vivo*

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ARTICLE INFO

Article history:
Received 3 May 2013
Available online 15 May 2013

Keywords:
Aurora-A kinase
Rho-kinase
Spaghetti squash
Myosin
Actin
Drosophila

ABSTRACT

Aurora-A kinase (AurA) is a key regulator of cellular processes involving microtubules. It has also been implicated in actin-dependent events, but the mechanisms that underlie the processes are not fully understood. Here we provide genetic and biochemical evidence suggesting that AurA negatively regulates Drok, the only known Rho-kinase orthologue in *Drosophila*. AurA directly phosphorylates Drok *in vitro*, and the overexpression of the nonphosphorylatable forms of Drok *in vivo* causes similar, but much stronger effects than that of wild-type Drok. The defects induced by the nonphosphorylatable forms of Drok are compensated by reducing the function of *myosin* downstream. Thus, phosphorylation of Drok by AurA normally suppresses Drok activity. We propose that AurA directly regulates actin-dependent processes by phosphorylating Rho-kinase.

1. Introduction

AurA is a highly conserved serine/threonine kinase, which was originally identified in *Drosophila* as functioning in centrosome separation and bipolar spindle formation [1]. The identification of AurA substrates has further demonstrated its role in regulating microtubule dynamics [2]. It is also known that the regulation of AurA expression or function is important in the maintenance of genomic integrity [2]. In addition to these microtubule-dependent events, few studies suggest that AurA plays roles in processes involving the actin cytoskeleton: the overexpression of AurA leads to cytokinesis failure in cultured cells [3], and *Drosophila* AurA is shown to be required for the asymmetric localization of Numb in sensory organ precursor cells [4] and larval neuroblasts [5–7]. Neuroblasts divide asymmetrically, enabling both self-renewal and differentiation into neurons or glia [8]. The asymmetric localization of cell fate determinants (Prospero (Pros), Brain tumor and Numb) and their adapters (Miranda and Partner of Numb (Pon)) are essential for the regulation of neuroblast proliferation [8,9]. Pharmacological studies indicate that Numb localization is an actin-dependent, and microtubule-independent, process [10]. In addition, it was reported that the phosphorylation of Par-6 by AurA initiated a phosphorylation cascade required for the asymmetric localization of Numb [7]. Thus, AurA has been suggested to be involved in the actin-dependent Numb localization process through regulating a particular component of the Numb localization machinery. However, it cannot entirely rule out the possibility that AurA may also play a direct role in regulating actin dynamics in some context.

Moreover, it has been reported that a vertebrate Rho-kinase isoform can be phosphorylated by AurA [11], although its functional significance and phosphorylation sites *in vivo* are unclear. In *Drosophila*, it is well established that Drok, the only known *Drosophila* Rho-kinase orthologue, plays critical roles in actomyosin-dependent processes, such as eye morphogenesis [12] and neuroblast asymmetric division [13]. In this study, given the possible connection between AurA and Rho-kinase, we investigated whether AurA can directly regulate Drok activity *in vivo*.

2. Materials and methods

2.1. Fly strains and genetics

$aurA^{ST}$, $aurA^{IM}$ and $aurA^{WK}$ lines were generated by imprecise excision of $aurA^{EY03490}$ line (Bloomington). All three mutants show non-complementation with previously known *aurA* mutants and deficiency lines that uncover the *aurA* gene locus. Single-fly PCR experiments were performed as previously described [14]. Oregon-R and yellow white strains were used as wild-type,

Abbreviations: AurA, Aurora-A kinase; Drok, *Drosophila* Rho-kinase; Sqh, spaghetti squash (Myosin II regulatory light chain); Zip, zipper (Myosin II).

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0006-291X
http://dx.doi.org/10.1016/j.bbrc.2013.05.028

aurA$^{87Ac\text{-}3}$ (Bloomington) and *aurA*^{287} (from D. Glover) as *aurA* alleles, *Df(3R)P-58* (Bloomington) and *Df(3R)Kar-D1* (Bloomington) as deficiency lines that uncover the *aurA* gene locus, *drok*^{2} as a *drok* null allele (Bloomongton) and *zip*^{1} as a strong *zip* allele (Bloomington) were used. For phenotypic analyses, we mostly used *aurA/Df(3R)Kar-D1* flies. *Spaghetti-squash* (*sqh*)*EE* and *sqhAA* lines were provided by R. Karess. *GMR-Gal4* was used to overexpress *UAS-Drok-WT*, *UAS-Drok-3A, UAS-Drok-2A and UAS-Drok-5A* (Fig. 4A) in the developing eyes. *UAS-aurA* transgene (from J. Knoblich) was expressed using *armadillo-Gal4* (from J.P Vincent) to rescue *aurA*ST, *aurA*^{IM} and *aurA*^{WK}.

2.2. Constructs

GST-Drok fusions were generated in pGEX5X-1 (Amersham-Pharmacia) and His-AurA was generated in pET45b(+)(Novagen). The mutants were generated using the QuickChange II site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. For transgenic flies, Drok-3A, Drok-2A and Drok-5A were cloned into pUAST. *UAS-Drok-WT* transgenic flies were provided by Nishida [15].

2.3. Kinase assay

The AurA expression construct (pET45b(+)-AurA) or only pET45b(+) vector was transformed into BL21-Gold (DE3) competent cells (stratagene) and purified using His·Bind Buffer Kit (Novagen) following the manufacturer's instructions. The protein fractions were further concentrated with Centricon (Millipore). The fraction processed from pET45b(+) vector-only transformation was used as a negative control (without AurA). Kinase assay was made in a kinase buffer (20 mM Hepes-Na, pH 7.4, 10 mM $MgCl_2$, 25 μM unlabeled ATP, 1 mM DTT and 2 μCi of [γ-^{32}P]-labelled ATP) at 30 °C. The reaction was stopped after 60 min by addition of SDS buffer. The boiled samples were submitted to SDS–PAGE analysis and autoradiography.

2.4. Immunohistochemistry

Larval brains and eye discs were fixed immediately after dissection in 4% paraformaldehyde for 20 or 30 min. Ovaries were processed as described [16] (Supplementary Fig. 1C and D). The antibodies used were: mouse anti- Discs large (Dlg) (1:40, Developmental Studies Hybridoma Bank) (Supplementary Fig. 1J–M), mouse anti-Miranda (1:50), rabbit anti-Pon (1:500, YN. Jan). Bio-Rad Radiance 2100 confocal microscope was used to acquire images and images were processed with Adobe Photoshop.

2.5. Antibodies

The N-terminus (residues 1–436) of Drok was cloned into pGEX5X-1 (Amersham-Pharmacia) to produce a GST fusion protein. Polyclonal antibodies were generated in rabbits by MBL (Nagoya, Japan). Rabbit anti-AurA (1:200, D. Glover) (Supplementary Fig. 1B), Rabbit anti-Drok (1:50) and mouse anti-α Tubulin (1:200, Sigma) (Supplementary Fig. 2B) were used for Western blot analysis.

3. Results

We first investigated whether AurA directly phospgorylates Drok. The His-tagged AurA protein and a series of Drok proteins fused with glutathione S-transferase (GST) (Fig. 1B) were used to determine whether Drok is a substrate of AurA. All of the N-terminal (Drok-N, residues 1–436), central (Drok-M, residues 420–855), and C-terminal (Drok-C, residues 842–1390) regions of Drok were phosphorylated by AurA *in vitro* (Fig. 1D). Along the Drok sequence, five serine residues (Ser 119, Ser 129, Ser 307, Ser 753 and Ser 908) match the consensus (R/K)-X-(S/T) motif or extended consensus (R/K)-X-X-(S/T) for AurA phosphorylation [2,17], and are also conserved between flies and humans (Fig. 1A). Replacement of these consensus Ser residues by alanine (Ala) revealed that each of the five serine residues in Drok is phosphorylated to different degrees by AurA *in vitro* (Fig. 1D and F); Ser 753 and Ser 908 appear to be only one phosphorylation site in Drok-M and Drok-C, respectively (Fig. 1D). The 3 serines in Drok-N contribute most of the phosphorylation signals of Drok-N (Fig. 1F and Supplementary Fig. 2A).

We next generated new *aurA* mutant alleles with various severities of phenotype, to facilitate genetic analyses. Three alleles, *aurA*ST, *aurA*^{IM}, and *aurA*^{WK}, were generated from a homozygous viable line *aurA*^{EY03490} by imprecise excision of a transposon (Fig. 2). The *aurA*ST mutation deletes nearly four-fifths of the *aurA* coding region from the 5′ UTR (Fig. 2 and Supplementary Fig. 1A). *aurA*^{IM} and *aurA*^{WK} deletions appear to remove some parts of the transposon and upstream of the start codon (Fig. 2 and Supplementary Fig. 1A). The *aurA*^{IM} and *aurA*^{WK} genes encode the wild-type AurA protein (Fig. 2 and Supplementary Fig. 1A), but their protein expression levels are strongly reduced, as revealed by Western Blotting (Supplementary Fig. 1B). The severity of the phentotypes of these three alleles varies as follows: *aurA*ST homozygotes die during prepupal to pupal stages; *aurA*^{IM} mutants die during eclosion, with a few escapers (females showing sterility were observed far fewer than male escapers); and *aurA*^{WK} mutants are mostly viable, but female-sterile). All three *aurA* alleles can be rescued from lethality by the expression of the *aurA* transgene driven by the *armadillo-Gal4* [18]. The surviving *aurA*^{IM} and *aurA*^{WK} adult flies show bristles containing two hairs (data not shown) as described before [4]. In addition, we observed slightly small and rough eyes in *aurA*^{IM} and *aurA*^{WK} mutants (Supplementary Fig. 1G and K), and abnormalities in *aurA*^{IM} mutant egg chambers, in which the ring canal structure [19] was occasionally twisted (Supplementary Fig. 1D), suggesting that AurA plays a general role in the regulation of actin cytoskeletons.

Using the new lines of *aurA* mutants, we tested whether *in vivo* functional relationship may exist between AurA and Drok, the only known Rho-kinase orthologue in the *Drosophila* genome [20,21]. In *Drosophila*, a single copy of the transgene encoding the constitutively active myosin II regulatory light chain (MRLC)/Spaghetti Squash (Sqh), in which phosphorylatable Ser 20 and Ser 21 are replaced by Glu (*sqhEE*) [22], can rescue the lethality caused by the *drok* null mutation [21], suggesting that *drok* activity can be monitored by *sqh* function downstream. We tested our hypothesis by introducing either one copy of *sqhEE* or an inactive counterpart (*sqhAA*, where the Ser 20 and 21 were replaced by Ala) into *aurA* mutant backgrounds. If reducing *aurA* function causes hyper-activation of Drok, Sqh must be hyper-phosphorylated in *aurA* mutants. Then, a further *sgh* activation by the introduction of *sqhEE* is expected to enhance *aurA* phenotypes. Indeed, introducing one copy of *sqhEE* into *aurA* backgrounds significantly enhances *aurA* mutant lethality (Fig. 3A), whereas two copies of *sqhEE* have no effect in the wild-type. Moreover, surviving *aurA*^{WK}/*sqhEE* flies show rougher eyes (Supplementary Fig. 1H and L) than do *aurA*^{WK} flies (Supplementary Fig. 1G and K). No such effects were observed when *sqhAA* was introduced into the various *aurA* backgrounds (data not shown). If the putative effect of AurA on Sqh phosphorylation is mediated by Drok, a reduction of *drok* activity may weaken the *sqhEE*-enhanced *aurA* phenotypes. We therefore examined whether the lethality caused by *sqhEE* in the *aurA* mutant backgrounds can be complemented by reducing endogenous *drok* activity. The null *drok*^{2} allele [21] was used to halve the genetic dose of *drok*. We observed that halving the genetic dose of *drok* suppresses

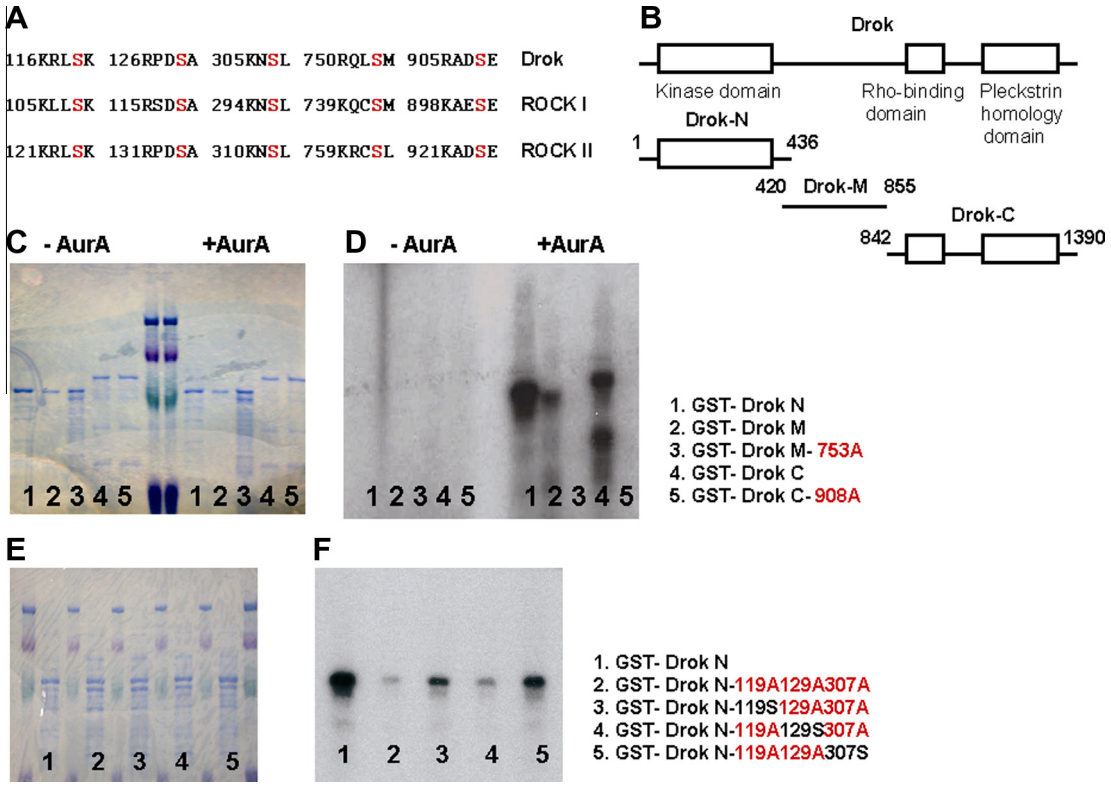

Fig. 1. AurA phosphorylates Drok *in vitro*. (A) Consensus and conserved sequences for AurA phosphorylation in Drok. Five consensus serines (red) are also conserved in the *Drosophila* and the human homologues. Numbers indicate first amino acid in each sequence. (B) Drok constructs used as substrates. (C, D) Kinase assay with or without recombinant AurA (+/−). Recombinant AurA phosphorylates GST-Drok-N, GST-Drok-M, and GST-Drok-C, but not mutants in which Ser 753 in GST-Drok-M or Ser 908 in GST-Drok-C is mutated to alanine. (E) and (F) Kinase assay of Drok-N. The phosphorylation signal of Drok-N is significantly reduced in case of all of Ser 119, Ser 129, and Ser 307 in Drok-N are mutated to alanine. Phosphorylated substrates were separated by SDS–PAGE, stained with Coomassie (C and E), and visualized by autoradiography (D and F).

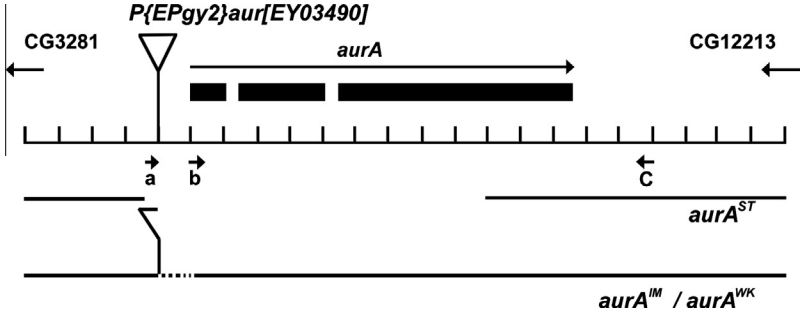

Fig. 2. Genomic organization of *aurA* region and molecular identity of the *aurA* mutations. Each division represents 120 bp of genomic DNA; the black boxes represent exons. The *P{EPgy2}aur[EY03490]* located122 bp upstream of the start codon. The positions of primers used for the excision screen are indicated by the arrows labeled a–c. $aurA^{ST}$ deletion removes the entire *P{EPgy2}aur[EY03490]* and the genomic sequence 168 bp upstream and 1075 bp downstream of the start codon, which includes most of the *aurA* coding region from the 5 UTR, leaving only the DNA sequences that code for a part of the C-terminal region from amino acid 318 (Supplementary Fig. 1A, left). $aurA^{IM}$ and $aurA^{WK}$ deletions appear to contain most parts of *P{EPgy2}aur[EY03490]* but remove some parts of *P{EPgy2}aur[EY03490]* and upstream of the start codon (Supplementary Fig. 1A, middle, right).

the enhanced lethality of $aurA^{WK}$ females bearing one copy of *sqhEE* (Fig. 3B), and ameliorates the enhanced rough eye phenotype of these flies (Supplementary Fig. 1I and M). When the dose of *myosin II/zipper* (*zip*) was reduced instead of *drok*, a similar but weaker effect was observed (Supplementary Fig. 1E).

To examine the role of Drok phosphorylation *in vivo*, we overexpressed wild-type Drok (Drok-WT) and its nonphosphorylatable forms in developing eyes using the UAS-Gal4 system [23]. Since Ser119, Ser129, and Ser 307 are within the kinase domain, and Ser 753 and Ser 908 are in the coiled-coil region, we tested three different mutant forms, in which either or both of these two groups of Ser were replaced by Ala (Drok-3A, Drok-2A, and Drok-5A) (Fig. 4A). Under the control of the *GMR Gal4* driver [24], overexpression of Drok-WT results in small, rough eyes (Fig. 4C), while Drok-3A and Drok-2A cause much smaller, rougher eyes (Fig. 4D and E), and Drok-5A results in pupal lethality, presumably due to its ectopic expression. These results suggest that these modified forms of Drok are hyperactivated. The eye phenotype generated by expression of Drok-WT, Drok-3A, or Drok-2A is suppressed by halving the copy number of *zip*, suggesting that *zip* function is enhanced downstream of these Drok hyperactive forms (Fig. 4G–I).

A

	Lethal phase (%)			Survival (%)	n
	Larval	Prepupal	Pupal		
$aurA^{ST}$, sqhEE	13	87	0	0	109
$aurA^{ST}$	0	81	19	0	112
$aurA^{IM}$, sqhEE	0	76	24	0	119
$aurA^{IM}$	0	24	67	9	118
$aurA^{WK}$, sqhEE	0	7	81	12	132
$aurA^{WK}$	0	0	7	93	104

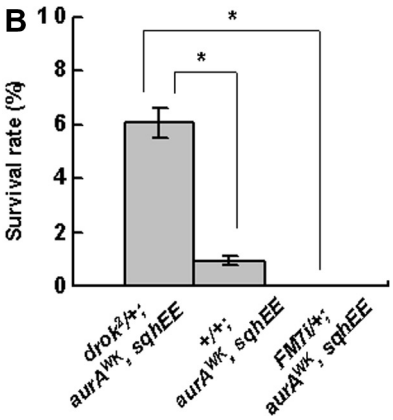

Fig. 3. AurA functions antagonistically to activity of the Drok signaling pathway. (A) One copy of *sqhEE* significantly enhances the *aurA* mutant phenotypes. Quantification of enhanced lethality by the presence of *sqhEE* in *aurA* mutants. A weaker *aurA* mutant bearing one copy of *sqhEE* shows a lethal phenotype similar to that of a stronger *aurA* mutant. (B) Quantification of the effect of halving the dose of *drok* on the lethality of $aurA^{WK}$/*sqhEE* mutants. Since *drok* is on the X chromosome, only survival rates (surviving flies/pupae) of the female mutants were compared (one-way ANOVA with Bonferroni; $^{*}P < 0.001$; error bars represent ± standard deviation). The survival rate of female mutants is much lower than that of the male mutants (A and Supplementary Fig. 1E).

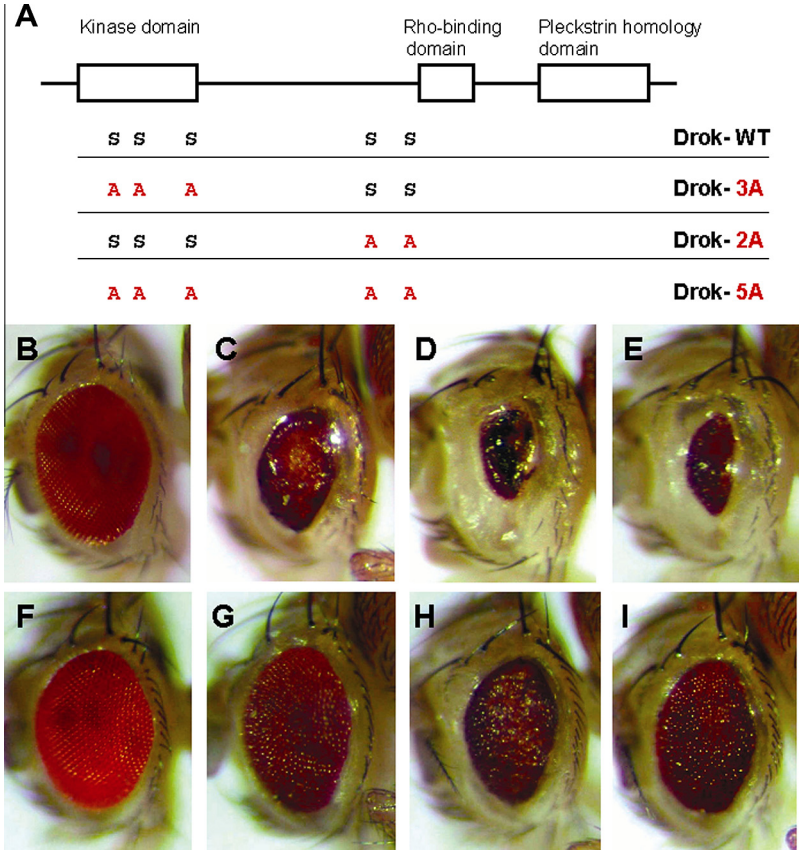

Fig. 4. Overexpression of the nonphosphorylatable forms of Drok causes similar, but much stronger effects than that of wild-type Drok in developing eyes. (A) Drok constructs used for overexpression. (B)–(E) Effects of overexpression of Drok mutants in the developing eye. Light microscopy photographs of eyes of (B) wild-type, (C) *GMR-Gal4/+; UAS-Drok-WT/+*, (D) *GMR-Gal4/+; UAS-Drok-3A/+*, (E) *GMR-Gal4/+; UAS-Drok-2A/+*, (F) *GMR-Gal4/+;* zip^{1}*/CyO*, (G) *GMR-Gal4/+; UAS-Drok-WT/*zip^{1}, (H) *GMR-Gal4/+; UAS-Drok-3A/*zip^{1}, and (I) *GMR-Gal4/+; UAS-Drok-2A/*zip^{1}. Much smaller and rougher eyes are observed with Drok-3A or Drok-2A overexpression, but reducing Zip activity rescues the eye phenotype generated by expression of Drok-WT, Drok-3A, or Drok-2A to similar levels.

4. Discussion

In this study, we demonstrated that AurA phosphorylates Drok to inhibit its activity *in vivo* by taking advantage of fly genetics. Based on our biochemical evidences that AurA can phosphorylate Drok *in vitro* (Fig. 1) and observations on the requirement for *aurA* in eye morphogenesis (Supplementary Fig. 1F and G) and ring canal structure (Supplementary Fig. 1C and D), we hypothesized that AurA may play a general role in the regulation of actin cytoskeletons by modulating the activity of Drok. In *Drosophila*, it has been known that the Rho-kinase (Drok)–MRLC/Sqh–Myosin II/Zip pathway functions in *Drosophila* eye morphogenesis [12] and

neuroblast asymmetric division [13]. Rho-kinase regulates myosin II activity through both activation of MRLC/Sqh and inhibition of myosin phosphatase that inhibits MRLC/Sqh [25,26]. In addition, Drok is reported to play a role in maintaining normal ring canal morphology [27] although it remains to be elucidated whether the regulation of MRLC/Sqh by Drok is also involved in maintaining normal ring canal morphology.

To examine our hypothesis, we used our new lines of *aurA* mutants (Fig. 2) and *sqhEE* [22]. Introducing one copy of *sqhEE* into *aurA* mutant backgrounds significantly enhances *aurA* mutant lethality (Fig. 3A) and rough eye phenotype of $aurA^{WK}$ (Supplementary Fig. 1G and H), indicating that constitutive phosphorylation of Sqh enhances *aurA* loss-of-function phenotypes. Furthermore, consistent with the idea that AurA normally antagonizes Sqh phosphorylation, halving the genetic dose of *drok* (Fig. 3B) or *zip* (Supplementary Fig. 1E) suppresses the enhanced lethality and rough eye phenotype of $aurA^{WK}$ females bearing one copy of *sqhEE* (Supplementary Fig. 1H and I). Taken together, these results suggest that AurA antagonizes the activity of the Drok-Sqh-Zip signaling pathway, namely, AuroraA ⊣Rho kinase → MRLC – Myosin + Actin. We also tested the effect of halving dose of *drok* in stronger $aurA^{IM}$ and $aurA^{ST}$ mutant backgrounds but could not find any clear suppression of the lethality in the mutant backgrounds (data not shown), presumably reflecting pleiotropic effects of *aurA* and relatively mild alteration of Drok activity.

Neuroblast asymmetric division in *Drosophila* is a representative actin-dependent process in which Rho kinase is involved [13]. Thus, we also examined asymmetric segregation of cell fate determinants in *aurA* mutants. We confirmed an increase in the brain size and neuroblast number (data not shown) in *aurA*-mutant phenotypes, which was reported as the consequence of mislocalization and/or lower levels of Numb [5,6]. Consistent with previous findings [5–7], $aurA^{ST}$ larval brains showed mislocalization of Pon. However, AurA seems not to be essential for the Miranda localization process (data not shown). Given the involvement of the same localization machinery (Par-6– atypical protein kinase C (aPKC) – Lethal (2) giant larvae (Lgl)) [8] induced by AurA in both Numb/Pon and Pros/Miranda localizations, this discrepancy can be simply explained by the notion that there may exist a yet unidentified AurA-independent mechanism of Miranda localization as previously described [7].

The functional significance of Drok phosphorylation *in vivo* was examined by overexpressing wild-type Drok and its nonphosphorylatable forms in developing eyes (Fig. 4). The overexpression of the nonphosphorylatable forms of Drok causes similar, but much stronger effects than that of Drok-WT (Fig. 4C–E). Furthermore, reduction of Zip activity suppressed the eye phenotype generated by expression of Drok-WT or the nonphosphorylatable forms of Drok (Fig. 4G–I). Taken together, these results suggest that the nonphosphorylatable forms of Drok are Drok hyperactive forms, and the enhancement of *zip* function is responsible for the defects induced by both wild-type Drok and the Drok hyperactive forms, supporting the idea that AurA phosphorylates Drok to inhibit its activity *in vivo*. Threfore, although the detailed mechanism by which the phosphorylated Drok affects the activity of its downstream targets remains to be elucidated, inhibitory phosphorylation of Drok is likely to be an important mechanism for regulating Myosin function *in vivo*.

In this study, we have proposed that AurA inhibits Rho-kinase by direct phosphorylation. Since Rho-kinase is known to regulate variety of actin-dependent processes, this model suggests a basic pathway by which AurA affects actin dynamics, and one that may explain the cytokinesis failure caused by AurA overexpression [3]. Inhibitory effects of AurA on Rho-kinase may act in other events.

Acknowledgments

We thank R. Karess, D. Glover, Y. Nishida, J. A. Knoblich, Y. N. Jan, Developmental Studies Hybridoma Bank and Bloomington Stock Center for sharing fly stocks and antibodies. Special thanks to Nao Ohta for making transgenic flies, Mutsuki Amano and Kozo Kaibuchi for advices and discussions about biochemistry, and Matsuzaki lab members for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.028.

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