Developmental Role of plk4 in *Xenopus laevis* and *Danio rerio*: implications for Seckel Syndrome

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID:	bcb-2015-0003.R1
Manuscript Type:	Article
Date Submitted by the Author:	29-Apr-2015
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Keyword:	polo like kinase, somite, lens, cell polarity, centrosome

Developmental Role of *plk4* in *Xenopus laevis* and *Danio rerio*: implications for Seckel Syndrome

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Running Header: A role for *plk4* in tissue organization?

Draft

Abstract

The *polo*-like kinases are a family of conserved serine/threonine kinases that play multiple roles in regulation of the cell cycle. Unlike its four other family members, the role of *Plk4* in embryonic development has not been well characterized. In mice, *Plk4*^{-/-} embryos arrest at E7.5, just prior to the initiation of somitogenesis. This has led to the hypothesis that *Plk4* expression may be essential to somitogenesis. Recently characterized human mutations lead to Seckel Syndrome. Riboprobe in situ hybridization revealed that *plk4* is ubiquitously expressed during early stages of development of *Xenopus* and *Danio*; in later stages, expression in frogs restricts to somites as well as eye, otic vesicle, and branchial arch, and brain. Expression patterns in fish remain ubiquitous. Both somite and eye development require planar cell polarity, and disruption of *plk4* function in frog by means of morpholino-mediated translational knockdown yields orientational disorganization of both these structures. These results provide the first steps in defining a new role for *plk4* in organogenesis and implicates a role in planar cell polarity, segmentation, and in recently described *PLK4* mutations in human.

Key Words: polo like kinase, somite, lens, centrosome, polarity

Introduction

Plk4 is a member of the *polo*-like family of serine-threonine kinases, which contain four other members, *Plk1/2/3/* and 5. These kinases are best known for their roles in cell cycle regulation and more specifically in mitotic progression and centrosome duplication (Sunkel and Glover 1988; Hudson et al. 2001; Bettencourt-Dias et al. 2005; Habedanck et al. 2005; Ko et al. 2005; Archambault and Glover 2009; Rosario et al. 2010).

Saccharomyces cerevisiae, possess only one *polo*-like gene, *cdc5*. *Drosophila* have at least two (*polo*, *sak*), while vertebrates such as *Xenopus* (*plk1-5*), fish (*plk1-4* with 2 versions of paralog 2), mouse (*Plk1-5*), and human (*PLK1-5*) have five.

Plk4 is highly expressed in the testes of adult mice as well as in the ovary, spleen, and thymus. During embryogenesis, murine *Plk4* is expressed during the proliferative stages of organ development, particularly in the liver, skin, thymus, somites, small intestine and kidney. In addition, *Plk4* expression is also seen in less proliferative tissues such as the nasal and olfactory mucosa and respiratory epithelium (Fode et al. 1994).

Mammalian mutants of *Plk4* display varied phenotypes. Murine *Plk4* null mutants die by day E7.5, just after gastrulation. They lack somites, notochord and neural tube even though neural crest develops (Hudson et al. 2001). Null mutant embryos have a normal population of trophoblast cells, however, the number of differentiated giant cells is reduced, commensurate with a lack of Hand1 localization to the nucleolus (Martindill et al. 2007). The inability of murine embryos to survive post gastrulation indicates a specific role for *Plk4* in the stages of development when organs begin to pattern and organize themselves. Both null and heterozygous murine cells exhibit a delay in mitotic progression (Hudson et al. 2001; Ko et al. 2005). In contrast to *Plk4*-null mice, heterozygote embryos develop normally without any externally obvious morphological phenotypes, however, elderly mice have an increased incidence of spontaneous liver and lung tumours indicating *Plk4* is haploinsufficient for tumour suppression. *Plk4*-null mice survive longer into development than *Plk1*-null mice which are embryonic lethal at the morula stage (Lu et al. 2008). By contrast, *Plk2*- null and *Plk3*-null mice are viable, so perhaps *Plk4*-null embryos survive through to gastrulation because of redundancy with

other *Plk* family members (Swallow et al. 2005).

In humans, mutation of either *PLK4*, or its interacting partner *CEP152*, causes Seckel Syndrome, a disorder associated with dwarfism, microcephaly, and abnormal eye, jaw, ear, and sometimes, spinal development (Kalay et al. 2011; Martin et al. 2014; Shaheen et al. 2014). In the case of *PLK4*, the dysfunction is only partial as the kinase domain remains intact. There are interesting phenotypic differences that can best be explained by understanding the subdomains affected and, we predict, the subcellular localization patterns in these different mutants. Finally, low viability of human blastocysts maps to an SNP in the domain containing PLK4 (McCoy et al. 2015).

In its N-terminal, like all other *polo*-like kinases, *Plk4* encodes a kinase domain responsible for phosphorylating protein substrates, and at the C-terminal, one canonical and two cryptic polo boxes that are thought to mediate protein-protein interactions and cellular localization (Leung et al. 2007). To date, *Plk4* has been characterized predominantly with regard to its importance in centrosome duplication: aberrant levels of *Plk4* protein result in the generation of supernumerary centrosomes and genome instability. For example, in tissue culture, PLK4 over-expression leads to the formation of supernumerary centrioles while loss of PLK4 leads to loss of centrosomes and mitotic defects (Bettencourt-Dias et al. 2005; Habedanck et al. 2005; Ko et al. 2005; Kleylein-Sohn et al. 2007; Rodrigues-Martins et al. 2007). Additionally, *plk4* can induce *de novo* centrosome formation in both *Drosophila* and *Xenopus* (Rodrigues-Martins et al. 2007; Eckerdt et al. 2011). Mutations that affect either its phosphorylation, partner-interacting, or subcellular localization characteristics also have phenotypic effects (Leung et al. 2002; Elia et al. 2003; Habedanck et al. 2005; Leung et al. 2007; Slevin et al. 2012).

PLK4 protein localizes to the centrosome during the cell cycle where it physically interacts with several components of the centrosome biogenesis machinery such as CEP152, CEP192, CDK11 and GCP6 and FBXW5 (Hudson et al. 2001; Leung et al. 2002; Hatch et al. 2010; Franck et al. 2011; Puklowski et al. 2011; Bahtz et al. 2012; Sonnen et al. 2013). Additionally, PLK4 has been shown to phosphorylate p53 and CHK2, both of which are important components of the DNA damage response (Ko et al.

2005; Petrinac et al. 2009). PLK4's role in other cellular compartments is less clear.

Although murine Plk4 predominately localizes to the centrosome, in G2 of the cell cycle it briefly moves to the nucleolus, an action that coincides with the differentiation of trophoblast stem cells (Hudson et al. 2001; Martindill and Riley 2008). Plk4 directly phosphorylates Hand1 in the nucleolus to influence trophoblast stem cell differentiation during mouse placental development (Martindill et al. 2007).

The lethality of *Plk4* null mice makes further study of its role in development difficult using this model system. We undertook to characterize developmental expression of *plk4* in *Xenopus* and *Danio*. We then used morpholino mediated translational knockdown to study the *plk4* loss of function phenotype in frog: we demonstrate a role for *plk4* in organizing tissues during organogenesis, and implicate a new function for *plk4* in localizing the centrosome to establish cell polarity, and potentially to link mitosis to the segmentation clock/oscillator.

Materials and Methods

Frogs

Xenopus laevis females were induced to ovulate with injection of 0.6-0.8cc of human Chorionic Gonadotrophin hormone (hCGH) (Intervet Canada Corp.). Embryos were obtained via *in vitro* fertilization and de-jellied in 2% cysteine (pH 8) as previously described (Drysdale and Elinson 1991). For wild type analysis embryos were left to develop in 0.1x MBS (88mM NaCl, 1mM KCl, 0.7mM CaCl, 1mM MgSO₄, 5mM HEPES pH7.8, 2.5mM NaHCO₃) at either 12°C or 17°C. Embryos to be used for morpholino injection were always incubated at 12°C post-injection. Embryos were staged according to (Nieuwkoop and Faber 1967), fixed in MEMPFA (0.1M MOPS pH 7.4, 1mM MgSO₄, 2mM EDTA, 4% paraformaldehyde) at 4°C overnight, and stored long term in 70% methanol.

Zebrafish

Adult zebrafish were organized in breeding systems with a male-to-female ratio of 1:2 in order to maximize spawning. Embryos were collected from the bottom of breeding tanks by siphoning (performed by Dr. Dennis Higgs, University of Windsor, Department of Biology), and then transferred to fresh carbon-treated tap water. Embryos were then dechorionated using Dumont #5 watchmaker forceps if they had reached a stage after initiation of pigmentation. Otherwise, dechoriation was performed after fixation to preserve embryo integrity. Embryos were fixed with 4% paraformaldehyde (PFA) dissolved in PBS-Tween overnight at 4°C. The next day, embryos were rinsed three times in 1x PBS, then dehydrated through an increasing methanol gradient for storage in 100% methanol at -20°C. They were processed for riboprobe in situ hybridization and probed at high stringency with zebrafish *plk4* (*pBluescript-SK⁻-zplk4* from Open Biosystems Inc) sense and antisense probes, in the manner described for frogs embryos

Cloning

The *Xenopus* probes were derived from *pCMV-SPORT6-Plx4* (Open Biosystems Inc.). A *plk4* truncation construct containing the first 1200bp of the *plk4* cDNA was cloned for riboprobe production. *Plk4-Sport6* was digested with *HindIII* to remove 2354bp from the 3' end of the *plk4* cDNA insert. The larger fragment, containing the Sport-6 vector backbone and the first 1200bp of *plk4* cDNA, was then re-ligated to circularize.

In Situ Hybridization

Staining to detect mRNA localization via *in situ* hybridization was performed according to standard protocols using digoxigenin-UTP (Roche) labeled probes that were hybridized at high stringency (68°C) (Sive et al. 2000). Embryos were imaged directly or cleared with BABB (2 parts benzoic acid: 1 part benzyl benzoate) prior to image collection using a Leica MZFLIII stereoscope and Northern Eclipse software (Empix, Canada).

Morpholino Design and Microinjection

For translational knockdown assays anti-sense morpholino oligonucleotides were designed and ordered through GeneTools, LLC (Orlando, USA). To confirm specificity two *plk4* specific morpholinos were designed to target different regions of the *plk4* transcript. *Plk4.upMO* sequence targeting the 5' UTR was 5'-TCTTTTCTCTCGCTGCTCGCGCCC-3' and *Plk4.IMO* sequence targeting the ATG start site was 5'-CTCTCTCCTATGCTGCCCGCCATGC-3'. The mis-match control sequence corresponding to *Plk4.1* was 5'-CTCTCTTCgTATcCTcCCCcCCATcC. A standard control morpholino targeted against the human β -globin gene was also ordered from GeneTools with sequence 5'-CCTCTTACCTCAGTTACAATTATA-3'. All morpholinos were tagged with a 3'-Carboxyfluorescein moiety for lineage tracing. Embryos to be used for morpholino microinjection were incubated briefly in 0.3x MBS and 2% Ficoll-400 (Sigma) prior to injection and then microinjected into their animal pole at either the 1 or 2 cell stage. Microinjections were performed using a Drummond nanoinjector maintained at a volume of 4.6nL. Post-injected embryos were incubated in 0.3xMBS and 2% Ficoll-400 at 12°C for at least 1.5 hours to allow healing. Embryos were subsequently moved into 0.1x MBS for incubation at 12°C degrees until they developed to appropriate stages for analysis. Experimental embryos were staged according Nieuwkoop and Faber (1967) and fixed in MEMPFA for either 2 hours at room temperature or overnight at 4°C.

Immunohistochemistry

Immunostaining was performed using standard protocols (Sive et al. 2000). Embryos were then incubated overnight at 4°C with primary antibodies to either anti- β 1 integrin (Drs. P. Hausen and V. Gawanta- 8C8, Developmental Studies Hybridoma Bank, Iowa City, USA) at 1:400 dilution, anti-12/101 (Dr. JP. Brockes-12/101, Developmental Studies Hybridoma Bank, Iowa City, USA) at 1:1000 dilution or, anti- γ - tubulin (Sigma GTU-88) at 1:100 dilution. Alexa-568 (Life Technologies) secondary antibody diluted 1:500 in PBT was used for β 1-integrin and γ -tubulin stained embryos. Anti-mouse-HRP secondary (Sigma) diluted to 1:200 in PBT was used for anti-12/101 embryos. Embryos stained secondarily with Alexa-568 were dehydrated to 70% methanol for later sectioning. Embryos stained with anti-12/101 were washed twice for 5 minutes with 1x PBS.

Antibody detection was achieved by addition of DAB (Sigma) diluted to 1mg/ml in PBT supplemented with 0.003% H₂O₂. The colour reaction was stopped by two five minute washes with PBT. Embryos were then dehydrated to 70% methanol and imaged as whole mounts or processed for sectioning and imaging.

Sectioning and Hoechst Stain

Sections were stained with Hoechst 33342 (Invitrogen) diluted 1:1000 and washed with PBST. Fluorescently stained sections were cover slipped with fluoromount G and imaged. Sectioned riboprobe in situ hybridized specimens that had been sectioned were also imaged on a Zeiss Axioscope fluorescent microscope using Northern Eclipse software.

Results

***Plk4* has a dynamic spatio-temporal pattern of expression during *Xenopus* development**

While the *Xenopus laevis* genome has only recently been sequenced, predictive analysis of *plk4* indicates a transcript with 16 exons, similar to what is found in mouse and human (mouse - accession NM 011495.2; human - accession NM 001190799.1; *Xenopus* - accession NM 001089677, *Xenopus* genomic sequence available at xenbase.org). Like its mammalian homologs, *Xenopus plk4* encodes both cryptic and the conserved *polo*-domains. One riboprobe was derived from full length *plk4* cDNA. The second probe was developed using only the first 1200bp of 5' sequence. Sense riboprobes were developed as controls for both probes.

In *Xenopus*, *plk4* is expressed ubiquitously in the early stages of development compared to sense controls (Fig. 1A-B). Expression begins to concentrate dorso-anteriorly in late gastrula until by stage 14, the anterior neural plate is particularly rich in transcript (Fig. 1C). At around stage 17 the *plk4* expression domain becomes more intense, particularly in the anterior neural plate, along the neural ridges, and then in neural tube, pre-somitic mesoderm and lateral plate mesoderm (Fig. 1D). At stage 20, expression restricts to the anterior somites, neural plate, the optic vesicles, and developing brain, so that by stage 24, when otic vesicle is forming, all these structures express *plk4* (Fig 1E-F). Faint

expression in the pre-somitic mesoderm (PSM) remains throughout the processes of segmentation as well as in newly formed somites (Compare sense control 1G to H in stage 30 embryos). In maturing somites, *plk4* expresses along the entire anterior-posterior length of the embryo as well as in developing lens and otic vesicle (Fig. 1I). *plk4* expression concentrates in somites predominantly dorsally in stage 22 embryos, and with a very slight central bias (Fig. 1J), in a location where the nuclei are seen to align after rotation. When looked at from the top at stage 26, there is still somitic expression (Fig. 1K). At stage 35, *plk4* expresses strongly in the lens vesicle although somewhat less so in developing retina, neural tube, notochord, and branchial arches (Fig. 1L). Sense probes produced a fainter signal than antisense, however it was consistent and present at high stringency and was produced in multiple replicates and hands.

In zebrafish, the gene is ubiquitously expressed from early through late stages of development (Figure 2A-H). Embryos incubated with the sense probe served as a negative control in these experiments, showing only background, non-specific staining compared to antisense-probed embryos.

Depletion of *Xenopus* *plk4* disrupts somite morphogenesis and patterning

Morpholino specificity was confirmed by four methods: morpholinos directed against two separate 5' sites produced identical results (Fig 3A); neither control mismatch nor standard control morpholinos produced these effects (Fig 3B); low combined concentrations of targeting morpholinos produced phenotypes consistent with the highest concentrations of either one alone (Table 1); *plk4* mRNA rescued a morphant eye phenotype (Fig 4). Somite phenotypes are elicited by both *plk4* over- as well as under-expression, and the consequent “rescue” combinations of mRNA and morpholino are difficult to interpret without ambiguity. Ectopic expression of Plk4 is known to induce supernumerary centrosomes in both frog and fly (Rodrigues-Martins et al., 2007; Eckerdt et al., 2011), so the competing and divergent effects are not without precedent. It is worth mentioning that some of the phenotypes that survived late into development, when heart normally starts beating, were similar to morphologies produced by off-target effects in fish – but this was expected since *plk4* interacts with both *hand1* and *p53*, and would

produce similar effects if acting as predicted: for example *hand1* mutant mice display heart and craniofacial anomalies (Firulli et al. 1998; Firulli et al. 2014). Additionally, *plk4* can induce *de novo* centrosome formation in both *Drosophila* and *Xenopus* (Rodrigues-Martins et al. 2007; Eckerdt et al. 2011). We have never seen this category of morpholino off-target effect in frog. Moreover, the morphologies obtained are consistent with mouse and human mutant phenotypes. We conclude that the action of the morpholino treatment is specific.

Examination of the gross morphology of *plk4MO* (morpholino) embryos that were unilaterally injected at the 2 cell stage (the uninjected side serves as a control) indicates that depletion of *plk4* protein results in a bent dorsal axis (Fig. 3C) and an eye phenotype (compare control Fig. 3D with morphant E). The eye phenotype ranged from diminished size (usually weakening ventrally first; Fig. 3E) to a complete absence of eye, and this latter was also associated with microcephaly. Ventrally diminished retinal pigmentation remained evident through to the late stage 30s, and this is as far as we followed the phenomena. A marker of lens placode formation, *pitx3*, can be completely inhibited by morpholino-mediated knockdown of *plk4* (Fig. 3F). The morpholino does not appear to inhibit expression of other markers that are expressed in retina, although the expression domains are altered. In the case of *pax6*, the domain is slightly diminished (Fig. 3G), and with *rax1*, inhibition in the optic vesicle following the lens placode stage is more severely curtailed (Fig. 3H). Among those embryos where placode is not completely abolished, eye development can still proceed to generate a relatively normal organ (Fig. 3I), albeit somewhat smaller than usual.

High doses of *plk4MO* (10ng) cause curvature of the dorsal axis that is the product of inhibited somitogenesis. Normally, pre-somitic mesoderm cells line up along the dorsal axis and then rotate 90° in cohorts of roughly 10-12 cells, however although such perpendicular cohorts emerge on the control side, the nuclei within the somitic mesoderm of the *plk4MO* injected side are randomly distributed. This is evident whether somitogenesis is monitored in whole mounts using antibody marker for somite myofibres (Fig. 3J, completely abolished in 87% of embryos), or in sections using Hoechst stain for nuclei (Fig. 3K-L). Control somites differentiate a morphology that is characterized by

defined borders and striated myofibre cells, however, even though the morphant side ultimately assumes a modicum of organization, somitic borders indicated by beta1-integrin remain poorly defined (compare the top control half with the morphant bottom of Fig 3K). Moreover, in morphants, the nuclei in somitic mesoderm do not organize, nor elongate normally (compare control to morphant sides in 3K-O), and gamma-tubulin stained centrioles fail to associate in a peri-nuclear fashion (compare Fig 3N to O). A similar failure of centrioles to associate with smaller nuclei was noted in lens placode as well. Embryos injected with *plk4MO* also appear to demonstrate impaired function of the segmentation clock: both *notch* and *hes4* banding patterns are inhibited on the morphant side (Fig 3P and Q respectively).

Discussion

Both *PLK4* and its interacting partner, *CEP152* have been identified as loci involved in the human disorder Seckel Syndrome (Kalay et al. 2011; Martin et al. 2014; Shaheen et al. 2014). In one instance, the encoded kinase domain remains intact, but the functions of both the cryptic and conserved *polo* domains, as well as two of three PEST motifs are abrogated (Shaheen et al. 2014). These deficits presumably interfere, at minimum, with the capacity of PLK4 to interact with partners like CEP152. Possibly, turnover of the protein could be impaired as well. In the other human mutation, the creation of an errant splice acceptor causes a missense mutation that interrupts the final and conserved *polo*-domain by premature termination (Martin et al. 2014). In this allele, all three PEST motifs as well as the cryptic *polo*-domains remain intact. The third and stereotypic *polo* domain is required for the protein to lose its auto-inhibition, and it is critical to stability and targeting for degradation (Klebba et al. 2015). Intriguingly, in zebrafish treated with morpholinos to inhibit translation of the last exon that contains the *polo*-box, the fish suffered a phenotype similar to that seen in human: foreshortened bodies, dorsal axis curvature, and reduced eye size (Martin et al. 2014). An additional defect in fish involved cardiac laterality defects. Cardiac defects have also been documented in Seckel patients (Can et al. 2010; Arslan et al. 2014).

Why, if the mammalian *PLK4* gene is expressed in a spatio-temporally restricted manner

and, as we show, fish *plk4* is expressed ubiquitously, are the phenotypes so similar? We speculate that both isoforms remain auto-inhibited, but can still bind with localized partners, such as CEP152, through their remaining and intact cryptic *polo* domain. This kinase-inhibited complex would produce an effect identical to the more severely truncated human mutant gene (Shaheen et al., 2014) that, lacking even the cryptic *polo* domain, would be unable to bind partners leaving them similarly unphosphorylated.

In our *Xenopus* study, the expression pattern of *plk4* corresponded well with patterns seen in mouse, however fainter antisense patterns posed a problem. There are no obvious repetitive sequences in *plk4*, but a sense signal would be consistent with reports of lncRNA in mouse Plk family members (Saito et al. 2011). Human *PLK4* is a likely site of intense miRNA complementarity – RepTar reports that 65 different miRNAs can bind human *PLK4* (Elefant et al. 2011). If present in sufficient quantity, they could bind probe to produce signal: for example, we can detect the short sequences of abundant antisense morpholino by similar means. The phenotypes of morphants are consistent with the lack of somites in null mutant mice, the eye phenotypes in morphant zebrafish (Martin et al. 2014), as well as with the craniofacial, eye, and otic/ear structures in human. So why was there no lethality in the human and zebrafish studies, and why did somitogenesis occur relatively normally in these organisms?

Our data from frog, as well as the null mutant mouse phenotypes are different from the human and zebrafish phenotypes in a substantial respect – morphants/mutants lack the functionality of a kinase domain as well as all three *polo*-like domains. In the human and zebrafish phenotypes, the kinase domain remains available and it presumably phosphorylates substrates near centrosomes where *PLK4* remains localized, or it may sequester binding partners thereby affecting their availability for downstream processes. In mouse and frog, the simultaneous absence of the kinase domain prohibits all other functions that *Plk4/plk4* has assumed over the course of evolution: in addition to the features normally associated with Seckel Syndrome, the animals also lose somites, and consequently a host of other structures necessary to embryonic viability. Why?

One important yet understudied function of the centrosome is its role in establishing

cellular polarity. The establishment of apico-basal polarity (ABP) is an important aspect of embryonic development, particularly in the formation of epithelial and glandular structures. Several changes occur during establishment of this polarity, one of which involves the re-positioning of the centrosome to the apical surface of the cell. In most epithelial cell types this allows for the formation of a non-centrosomal, apical-basal microtubule array and primary cilia (Musch 2004; Satir and Christensen 2007; Tang and Marshall 2012). Polarization of the microtubule array within the apico-basal axis allows for vesicular transport between apical and basal ends of the cell (Musch 2004). The centrosome is polarized to the apical surface during neural, lens, retinal, mammary, intestinal and cochlear development (Musch 2004; Manning et al. 2008; Tang and Marshall 2012). Moreover, PLK4 mutation results in ciliopathies (Martin et al., 2014). Presumably, centrosome polarity is important to other developmental processes that require the coordinated movement of whole cohorts of cells: among these would fall the elongation, orientation, and alignment of cells that is prerequisite to the formation of somites and lens placode.

In both mammals and frog, lens placode formation represents a first step in the induction of the tissues that form the eye. Mutations that inhibit lens placode development in mammals result in smaller eyes or aniridia (Semina et al. 1998; Semina et al. 2000). In frogs, the absence of placode leads to complete abolition of retinal induction and the eye fails to form (Khosrowshahian et al. 2005). Clearly, inhibition of *plk4* translation in frogs is sufficient to inhibit lens placode formation (as monitored by expression of *pitx3*) and has dire consequences for the development of eye.

Although murine and amphibian somitogenesis appear to be affected in a similar manner by *Plk4/plk4* inhibition, the usual process of segmentation differs substantially between the two. In amniotes, the somites bud off and epithelialize, whereas in frogs, cells of the presomitic mesoderm align longitudinally along the dorsal axis, and then rotate in cohorts of 10-12 cells to rest perpendicular to the neural tube (Hamilton 1969). In our study of frog somitogenesis, *plk4* dysfunction disrupts this alignment of cells both in the pre-somitic mesoderm as well as in the regions normally filled with maturing somites: centrosomes appear scattered, and nuclei fail to elongate. Other markers of somite

maturation were also perturbed including the deposition of beta-integrin that separates somites. Taken in conjunction with the lens placode phenotype, this might suggest a role for *plk4* in planar cell polarity. Finally, it is interesting to note that not only was somitogenesis mechanically disrupted, but elements of the segmentation clock (*notch* and *hes4*) were also impaired: a previous study has linked the genetic network that embodies the segmentation oscillator/clock to the timing of cytokinesis (Delaune et al. 2012), so it will be interesting in future to query the extent to which Plk4 mediates or coordinates segmentation oscillators with cell division.

Acknowledgements

Thanks are due to: Drs Zuber, Harris, Kintner, and the NIBB/NIG/NBRP *Xenopus laevis* EST project for the gift of plasmids; Dr. Dennis Higgs for the gift of zebrafish embryos; Drs. P. Hausen and V. Gawanta via the Developmental Studies Hybridoma Bank (Ames Iowa) for antibody. The technical assistance of Dr. A. Ward and Ms A. Malysa are acknowledged. The Natural Sciences and Research Council (NSERC) of Canada is thanked for funding of Discovery Grants to MJC (203459) and JH (298476). Thanks are also due to NSERC and OGS for scholarships to NP and CER.

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	Phenotype & Number	plkMO1 10 ng	plkMO2 10 ng	plkMO1 + plkMO2 1 ng	CMO 20 ng
Eyes Scored Stages 34-37	n =	44	124	17	143
	Small Eye	27%	25%	0%	13%
	Dorso/Ventral	36%	25%	35%	17%
	No Eye	36%	67%	65%	0%
Somites Scored Stages 22-26	n =	54	66	22	174
	Curved Axis	83%	88%	100%	10%
	Somites Inhibited	71%	79%	91%	11%

Table 1

Table 1. Morpholino phenotype specificity. Data comparing high dose targeting morpholino (10 ng *plkMO 1* and 2) phenotypes with low dose combined (1 ng *plkMO 1* and 2) and high dose control morpholino experiments (20 ng CMO).

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Figure Legends

Figure 1. Developmental expression patterns of *Xenopus plk4* revealed by riboprobe insitu hybridization. Embryos display ubiquitous expression of *plk4* in early blastula (compare sense control A with antisense probe B). *Plk4* expression is high in anterior neural plate (ANP) by stage 14 (C), and in anterior neural ridge (ANR) and neural folds (NF) by stage 17 (D). Over the span of early organogenesis (E-F), expression is high in brain (B), neural tube (NT), optic placode (OpP), and otic vesicle (OtV), and subsequently in eye structures, otic placode (OtP), somites (S), and pre-somitic mesoderm (PSM). Stage 30 embryos show expression in somites (S), developing retina (R), lens placode (LP) and branchial arches (compare sense control G to antisense H). Although expression remains high in head and somites, *plk4* downregulates throughout the rest of stage 33 embryos (I). In cross sections (J and K), expression localizes to somites (S), and to neural tube (NT), retina (R), lens (L), but is absent from surrounding integument in (L) (bars 40 μ m).

Figure 2. Zebrafish *plk4* is ubiquitously expressed. Riboprobe wholemount in situ hybridization shows expression is ubiquitous at all stages of zebrafish development (compare sense control A,C,E, and G with B, D, F, and H. Darker outlines of developing organs in H are optical edge effects and are not specific.

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Figure 3. Phenotypes of morpholino-mediated translation knockdown of *plk4* in *Xenopus*. Diagram (A) illustrates placement of the antisense morpholinos relative to the ATG start site of *plk4*. External morphant morphologies include a bent dorsal axis and eye anomalies (compare controls B and D with morphants C and E). Eye phenotypes ranged from absent, to diminished size, to dorso-ventral patterning defects. Ventral eye development tended to be affected most easily, and this was reflected by abnormal development of pigmented retina (black arrow, Fig. 3 E). Embryos unilaterally injected at the 2 cell stage yield defects detectable by probes for eye development such as *pitx3* in lens placode, *pax6* and *rax1* in developing eye field (black arrow - uninjected side, white arrow - morpholino injected side in F, G and H respectively). Embryos are picture face-forward from the front. If lens placode was not entirely prohibited, optic induction occurred and produced relatively normal eye structures (I: L – lens, R – retina). Dorsal curvature resulted from abnormal somite development as revealed by the absence of normal patterns of myogenic markers (J: black arrow - uninjected side, white arrow - morpholino injected side), or oriented cohorts of pre-somitic cells (compare hoechst stained nuclei in uninjected side – white arrows, compared to disorganized cells below in K), and partitioning of somites is clear and discrete as indicated by borders staining red (arrows) for $\beta 1$ integrin on the control side, but is rudimentary, if at all present, on the morphant side (K). At lower magnification the Hoechst-stained nuclei are neatly aligned (white arrows) and cells on the morphant side more obviously disorganized (L) and scattered. In presomitic mesoderm the alignment and stacking of elongate nuclei is ordered on the control side verses the morphant side where nuclei are smaller and fall out of alignment (compare upper control boxed nuclei in (M) to lower boxed morphants). At higher magnification, immunohistochemistry reveals that gamma-tubulin labelled centrioles (arrows) associate with ordered files of elongated nuclei in control somites (N) but among smaller, disordered nuclei on the morphant side (O) (bars – 20 μ m). Segmentation clock markers *notch* and *hes4* are both inhibited by *plk4* dysfunction (black arrow - uninjected side, white arrow - morpholino injected side in P and Q).

Figure 4. Graph of lens phenotypes and rescue of morpholino phenotype by coinjection with *plk4* mRNA. Eye placode presence and morphology was assessed using in situ hybridization of marker *pitx3*. Severe morpholino phenotypes (lens absent) could be partially rescued by co-injection of *plk4* mRNA with morpholino. This pushed more eyes from the “absent” category into the “small lens” or “normal” categories. Figure represents summed data of experiments in triplicate. SCMO is control morpholino. * $p \geq .001$ difference between 10 ng dose morpholino alone and 10 ng morpholino plus rescue mRNA. ** $p \geq .001$ difference between 5 ng dose morpholino and 10 ng morpholino plus rescue mRNA.

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Developmental expression patterns of *Xenopus plk4* revealed by riboprobe in situ hybridization. Embryos display ubiquitous expression of *plk4* in early blastula (compare sense control A with antisense probe B). *Plk4* expression is high in anterior neural plate (ANP) by stage 14 (C), and in anterior neural ridge (ANR) and neural folds (NF) by stage 17 (D). Over the span of early organogenesis (E-F), expression is high in brain (B), neural tube (NT), optic placode (OpP), and otic vesicle (OtV), and subsequently in eye structures, otic placode (OtP), somites (S), and pre-somitic mesoderm (PSM). Stage 30 embryos show expression in somites (S), developing retina (R), lens placode (LP) and branchial arches (compare sense control G to antisense H). Although expression remains high in head and somites, *plk4* downregulates throughout the rest of stage 33 embryos (I). In cross sections (J and K), expression localizes to somites (S), and to neural tube (NT), retina (R), lens (L), but is absent from surrounding integument in (L) (bars 40 μ m).
279x215mm (300 x 300 DPI)

Phenotypes of morpholino-mediated translation knockdown of *plk4* in *Xenopus*. Diagram (A) illustrates placement of the antisense morpholinos relative to the ATG start site of *plk4*. External morphant morphologies include a bent dorsal axis and eye anomalies (compare controls B and D with morphants C and E). Eye phenotypes ranged from absent, to diminished size, to dorso-ventral patterning defects. Ventral eye development tended to be affected most easily, and this was reflected by abnormal development of pigmented retina (black arrow, Fig. 3 E). Embryos unilaterally injected at the 2 cell stage yield defects detectable by probes for eye development such as *pitx3* in lens placode, *pax6* and *rax1* in developing eye field (black arrow - uninjected side, white arrow - morpholino injected side in F, G and H respectively). Embryos are picture face-forward from the front. If lens placode was not entirely prohibited, optic induction occurred and produced relatively normal eye structures (I: L - lens, R - retina). Dorsal curvature resulted from abnormal somite development as revealed by the absence of normal patterns of myogenic markers (J: black arrow - uninjected side, white arrow - morpholino injected side), or oriented cohorts of pre-somitic cells (compare hoechst stained nuclei in uninjected side - white arrows, compared to disorganized cells

below in K), and partitioning of somites is clear and discrete as indicated by borders staining red (arrows) for β 1 integrin on the control side, but is rudimentary, if at all present, on the morphant side (K). At lower magnification the Hoechst-stained nuclei are neatly aligned (white arrows) and cells on the morphant side more obviously disorganized (L) and scattered. In presomitic mesoderm the alignment and stacking of elongate nuclei is ordered on the control side versus the morphant side where nuclei are smaller and fall out of alignment (compare upper control boxed nuclei in (M) to lower boxed morphants). At higher magnification, immunohistochemistry reveals that gamma-tubulin labelled centrioles (arrows) associate with ordered files of elongated nuclei in control somites (N) but among smaller, disordered nuclei on the morphant side (O) (bars – 20 μ m). Segmentation clock markers notch and hes4 are both inhibited by plk4 dysfunction (black arrow - uninjected side, white arrow - morpholino injected side in P and Q).
215x279mm (300 x 300 DPI)

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Lens Phenotype (*pitx3* ISH)