Sea urchin *akt* activity is Runx-dependent and required for post-cleavage stage cell division

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Summary

In animal development following the initial cleavage stage of embryogenesis, the cell cycle becomes dependent on intercellular signaling and controlled by the genomically encoded ontogenetic program. Runx transcription factors are critical regulators of metazoan developmental signaling, and we have shown that the sea urchin Runx gene *runt-1*, which is globally expressed during early embryogenesis, functions in support of blastula stage cell proliferation and expression of the mitogenic genes *pkc1*, *cyclinD*, and several *wnts*. To obtain a more comprehensive list of early *runt-1* regulatory targets, we screened a *Strongylocentrotus purpuratus* microarray to identify genes mis-expressed in mid-blastula stage *runt-1* morphants. This analysis showed that loss of Runx function perturbs the expression of multiple genes involved in cell division, including the pro-growth and survival kinase Akt (PKB), which is significantly underexpressed in *runt-1* morphants. Further genomic analysis revealed that Akt is encoded by two genes in the *S. purpuratus* genome, *akt-1* and *akt-2*, both of which contain numerous canonical Runx target sequences. The transcripts of both genes accumulate several fold during blastula stage, contingent on *runt-1* expression. Inhibiting Akt expression or activity causes blastula stage cell cycle arrest, whereas overexpression of *akt-1* mRNA rescues cell proliferation in *runt-1* morphants. These results indicate that post-cleavage stage cell division requires Runx-dependent expression of *akt*.

Key words: Runx, Akt, PKB, Sea urchin embryo, Development, Cell proliferation

Introduction

In animals the somatic cell cycle is non-autonomous, subjugated to the developmental program encoded in the genome as a gene regulatory network (GRN) that both controls and is responsive to intercellular signaling (Davidson, 2006). This manifests during ontogeny following the initial cleavage stage of embryogenesis, when cell proliferation comes under control of the zygotic genome and occurs in lineage- and/or tissue-specific patterns. The genomic control of cell cycle development is mediated by regulatory circuitry that controls the transcription of numerous cell cycle genes, such as D-type cyclins, various cyclin dependent kinase (cdk) inhibitors, and checkpoint kinases, that in turn control G1 to S or G2 to M transitions. Deciphering the GRN that controls cell cycle development thus requires identification of relevant transcriptionally-controlled cell cycle genes and their regulators.

The Runx family of sequence-specific DNA binding proteins are key transcriptional regulators of cell proliferation and differentiation, and are essential for animal development (reviewed by Coffman, 2003; Coffman, 2009). Runx genes have been referred to as “master control genes” owing to the rate-limiting roles that they play in the developmental differentiation of specific cell lineages, and as both “oncogenes” and “tumor suppressors” in reference to the neoplastic effects associated with both gain and loss of Runx function (Cameron and Neil, 2004; Ito, 2008; Ryoo et al., 2006). However, such labels oversimplify the developmental role of Runx, which is context-dependent and hence resistant to functional categorization in simple reductionist terms. The three vertebrate Runx paralogues are expressed in complex and overlapping developmental patterns, affording redundancy and/or potential for functional compensation, and it is now clear that Runx is sometimes if not always required both for the proliferation of progenitor cells as well as the differentiation of their descendents (Coffman, 2003; Coffman, 2009).

In vivo developmental studies of relatively simple experimental models such as fruit flies, nematode worms and sea urchin embryos suggest that Runx occupies a unique functional niche in the cell physiology of animal development, wherein cell growth, proliferation and survival depends on intercellular signaling (Coffman, 2003; Coffman, 2009; Kagoshima et al., 2007; Nimmo and Woollard, 2008). One emerging generalization is that Runx is a linchpin for such signaling, interacting at multiple levels with each of the major signal transduction pathways to help coordinate developmental transitions (Coffman, 2009). This involves cooperative physical interactions between Runx proteins, signal-transducing transcription factors (e.g. Smads, TCF, Ets, nuclear receptors,

etc.), chromatin modifying enzymes, and nuclear architecture, as well as gene regulatory network circuitry wherein Runx controls the expression of genes required for cell signaling and vice versa (reviewed by Coffman, 2009). Thus, in some circumstances Runx may function as a single rate-limiting switch between alternate cell fates (exerting "master control"), while in others (and perhaps more commonly) it is necessary but not sufficient for specification of a given cell fate. The context-specificity of Runx function applies not only to cell, tissue, and organism type, but also to developmental stage. Hence, like a number of other transcription factors, in some contexts Runx may provide a "toggle switch", repressing a gene at one stage of development, and activating that same gene at another stage, which involves context-dependent recruitment of co-repressors such as Groucho and co-activators such as CBP.

Embryos of the sea urchin *Strongylocentrotus purpuratus* normally express only one of the two Runx genes encoded in the genome of that species, namely *runt-1*. At early blastula stage *runt-1* is expressed throughout the embryo and later (beginning at gastrula stage) it becomes confined to those lineages wherein cells continue to proliferate (Robertson et al., 2002). When *runt-1* expression is blocked using morpholino-antisense oligonucleotides, embryos arrest development at late blastula stage owing to widespread apoptosis (Coffman et al., 2004; Dickey-Sims et al., 2005), which is preceded by impaired cell proliferation (Robertson et al., 2008). Prior to or concomitant with these defects, *runt-1* morphants underexpress several *wnt* genes, including the key endomesodermal genes *wnt8* and *wnt6*, as well as genes whose products function cell autonomously to promote cell proliferation and/or survival, including *pkc1* (which encodes the single conventional protein kinase C in sea urchins) and *cyclinD* (which encodes the single D-type cyclin of sea urchins) (Coffman et al., 2004; Dickey-Sims et al., 2005; Robertson et al., 2008). Thus sea urchin *runt-1* is required for the activation of multiple genes involved in mitogenic and survival signaling beginning at blastula stage.

To obtain a more comprehensive view of *runt-1* function during its initial phase of expression we used a microarray to identify genes that are mis-expressed in blastula stage *runt-1* morphants. Numerous genes were found to be either underexpressed or overexpressed. The former set included one of two *S. purpuratus* genes that encode Akt/PKB (protein kinase B), a well-known mediator of growth and survival signaling in animals. Here we provide the initial published characterization of both sea urchin *akt* genes, *akt-1* and *akt- 2*. We show that blastula stage accumulation of their mRNAs is Runt-1-dependent, and that their activity is required for continuation of cell division after cleavage stage. Thus *akt-1* and *akt- 2* are part of the Runx-dependent battery of genes that promote somatic cell proliferation during sea urchin embryogenesis.

Results

Akt expression is Runx-dependent in the sea urchin embryo

A custom Agilent microarray (described in Materials and Methods) was used to identify genes regulated by the sea urchin (*Strongylocentrotus purpuratus*) Runx protein Runt-1 at 18 hrs post-fertilization (hpf), which is hatching blastula stage. At this stage of development the cell cycle has transitioned to zygotic control (Kelso-Winemiller et al., 1993), and *runt-1* mRNA is globally expressed at about half-maximal per-embryo levels (Coffman et al., 1996; Robertson et al., 2002) (Fig. 1A).

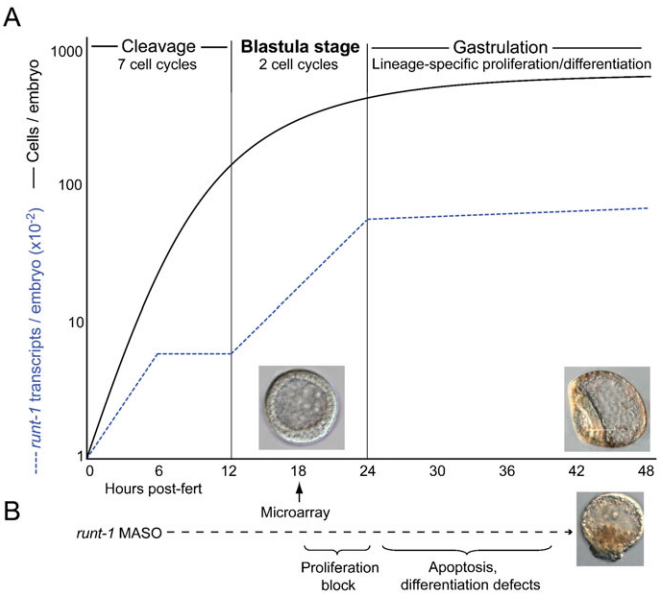


Fig. 1. Summary of the effects of Runt-1 knockdown in relation to the temporal pattern of cell proliferation and *runt-1* expression in the sea urchin embryo. (**A**) The canonical temporal pattern of cell proliferation during sea urchin embryogenesis (black curve) (adapted from Davidson, 1986), related to the temporal pattern of *runt-1* transcript accumulation (dotted blue line) (adapted from Coffman et al., 1996). *Runt-1* transcripts accumulate 10-fold (per embryo) from early to late blastula stage, i.e. between 12 and 24 hours post-fertilization (hpf) (Coffman et al., 1996). (**B**) Effects of Runt-1 knockdown. The first obvious effect in *runt-1* morphants consists of impaired cell proliferation between 18–24 hpf (Robertson et al., 2008); subsequently the morphants exhibit widespread apoptosis and differentiation defects (Coffman et al., 2004; Dickey-Sims et al., 2005). The gene expression microarray analysis (NCBI GEO accession number GSE19751) was performed on 18 hr embryos.

Embryos in which this expression is blocked by morpholino-antisense oligonucleotide (MASO)-mediated knockdown display impaired cell proliferation beginning at 18 hpf (Robertson et al., 2008) (Fig. 1B). We thus reasoned that gene expression changes underlying the proliferation block would be detectable at 18 hpf, and that the majority of the genes identified as being underexpressed would be direct targets of Runt-1. Genes identified as overexpressed on the other hand might be expected to include both direct and indirect targets, as many maternal mRNAs undergo rapid blastula stage decay (Davidson, 1986; Kelso-Winemiller et al., 1993), and it is possible that Runt-1 activates one or more genes required for this process.

The screen identified 68 genes that were consistently underexpressed (supplementary material Table S1) and 89 genes that were consistently overexpressed (supplementary material Table S2) by a factor of at least two. Many of these genes are known to function in cell division. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurements using primers specific to a subset of these genes confirmed that blastula stage *runt-1* morphants underexpress *akt* (Protein Kinase B, see below, Fig. 2), as well as *grb2* (Growth Factor Receptor Bound 2 protein) and *wrn/recql4* (RecQ Helicase). Genes confirmed by qRT-PCR to be overexpressed included *foxy* (Forkhead c-like transcription factor) and *nsd2* (Nuclear receptor-binging SET domain-containing protein).

In animal development Akt-mediated signaling promotes both the proliferation and survival of cells (Buttrick et al., 2008;

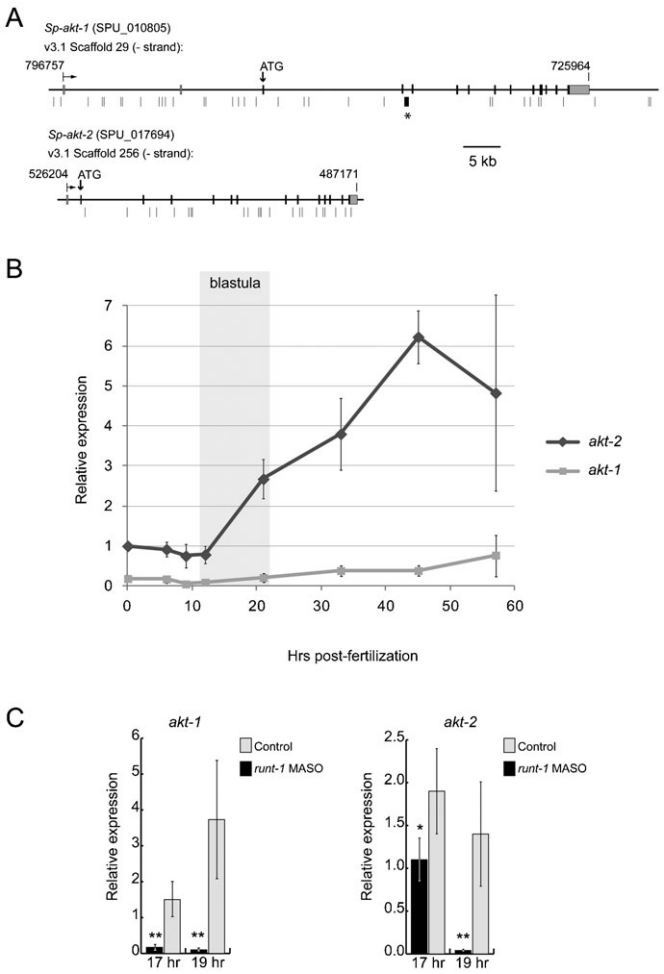


Fig. 2. *Sp-akt-1* and *Sp-akt-2* gene schematics and mRNA levels over developmental time in normal and *runt-1* morphant blastulae. (**A**) Schematic of the *Sp-akt-1* and *Sp-akt-2* loci from build 3.1 of the *S. purpuratus* genome, with exons depicted as boxes (non-coding grey, coding black), and putative Runx target sites matching the consensus $TG^{T}/_{C}GGT$ (or its reverse complement) marked as thin lines below. A 600 bp repeat sequence in intron 4 (*) contains a tandem array of 56 potential Runx target sites (see supplementary material Fig. S2). (**B**) Relative transcript levels from both genes at eight time points spanning embryogenesis, with maternal *akt-2* set to 1. Error bars represent the standard deviations of replicate measurements. (**C**) Relative *akt-1* and *akt-2* mRNA levels in *runt-1* morphants (black bars) compared to controls (grey bars) in two different batches of experimental embryos, one collected at 17 hpf (average of four replicate measurements ± the standard deviation), the other at 19 hpf (average of three replicate measurements ± standard deviation). Relative levels are with respect to levels measured in normal 12 hr embryos in the experiment depicted in (B). The fact that *akt-2* transcript levels are somewhat higher and much less strongly affected at 17 hrs than at 19 hrs most likely reflects biological variability between the batches of embryos (obtained from different outbred crosses). This amount of biological variability in expression levels is not unusual in sea urchins.

Lawlor and Alessi, 2001; Peng et al., 2003; Vivanco and Sawyers, 2002), both of which are impaired in *runt-1* morphants (Fig. 1B). Using the sea urchin genome resource SpBase (http://www.spbase.org) (Cameron et al., 2009) we determined that sea urchin Akt, which has not been previously characterized, is encoded in the *S. purpuratus* genome by two genes, which we have designated *Sp-akt-1* and *Sp-akt-2* (Fig. 2A; supplementary material Fig. S1). The sequences of the two proteins are more similar to one another (67% identity, 79% similarity) than to any other Akt homologue, suggesting that the two genes are derived from an echinoderm-specific duplication. Among the human isoforms, Akt-3 exhibits the highest similarity to both of the sea urchin sequences, being more similar to Sp-Akt-1 (65% identity) than to Sp-Akt-2 (59% identity).

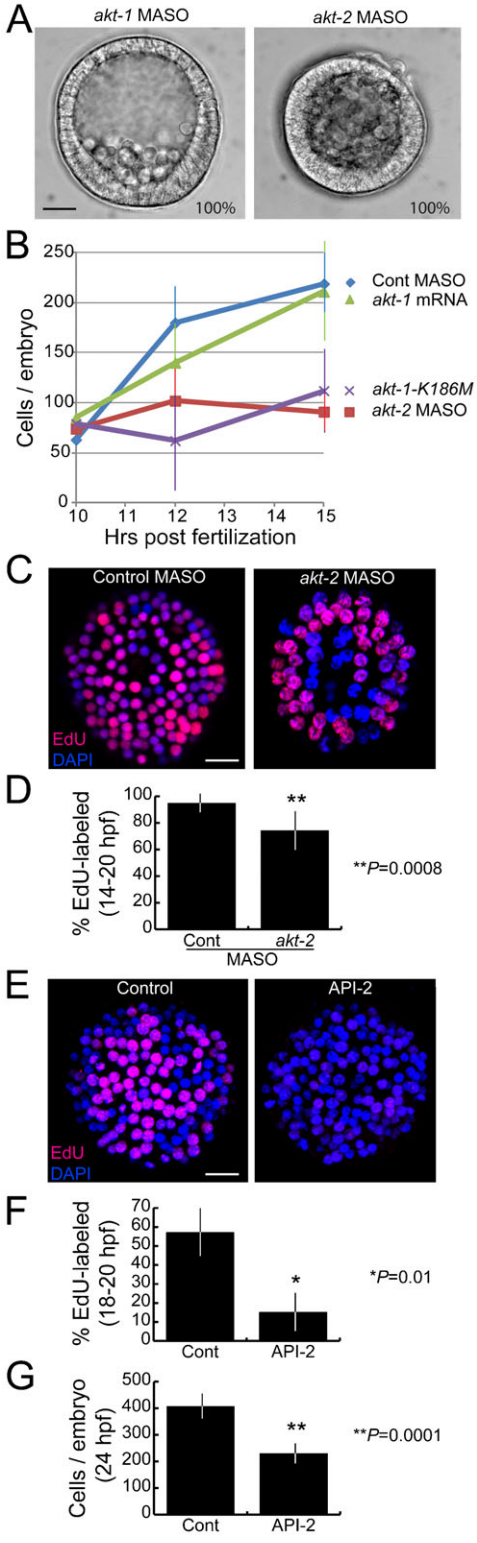
To define the temporal pattern of Akt expression, we carried out qRT-PCR measurements of *akt-1* and *akt-2* transcript levels over a time-course of normal development through early pluteus stage (Fig. 2B). *Akt-1* transcripts are present at constant levels in the egg and early cleavage stages, decline somewhat at morula stage, and then begin to accumulate during blastula stage (between 9 and 24 hpf, Fig. 2B), paralleling the accumulation of *runt-1* trancripts over the same interval (Fig. 1A) (Coffman et al., 1996). *Akt-2* transcripts are about 5–10-fold more abundant than those of *akt-1*, and also begin accumulating during blastula stage, reaching a maximum (estimated to be ~250,000 transcripts per embryo) by late gastrula stage. In *runt-1* morphants, the blastula stage accumulation of both *akt-1* and *akt-2* transcripts is impaired, as shown for each gene by two experimental measurements (biological replicates), one in 17 hr embryos and the other in 19 hr embryos (Fig. 2C). In sum, these results show that the transcripts of both *akt-1* and *akt-2* accumulate during blastula stage, and that this accumulation depends on *runt-1* expression.

Interrogation of the genomic sequence corresponding to *Sp-akt-1* and *Sp-akt-2* revealed numerous instances of the consensus Runx binding sequence $TG^{T}/_{C}GGT$ (in reverse complement $ACC^{A}/_{G}CA$) (Fig. 2A), suggesting that both are likely to be direct Runt-1 targets. Remarkably, intron 4 of the *akt-1* locus contains a 600 bp repetitive sequence element bearing a tandem array of 56 Runx consensus binding sequences (ACCACA, Fig. 2A, asterisk; supplementary material Fig. S2). A scan of the *S. purpuratus* genome using a sliding 600 bp window showed that this is the highest density of potential Runx sites in the vicinity (within 10 kb) of any annotated gene in the genome. By itself this repetitive sequence fails to activate a GFP reporter gene containing a basal promoter (data not shown), indicating that it does not function as an enhancer. While its functionality (if any) is not known, repetitive DNA containing tandem arrays of transcription factor binding sites have been found in some cases to function by locally sequestering the transcription factors that bind those sites (Liu et al., 2007). Further work involving chromatin immunoprecipitation and *cis*-regulatory reporter gene analysis is required to determine which (if any) of the putative target sites identified in each *akt* locus binds Runt-1 and mediates Runx-dependent transcriptional activity.

Akt is required for blastula stage cell division

Given the well-known role of Akt in mitogenic signaling in both normal and neoplastic growth, we hypothesized that the observed loss of *akt* expression in *runt-1* morphants might contribute to the impaired cell proliferation observed in those embryos. To test this we examined the effects of morpholino antisense oligonucleotides (MASOs) designed to block translation of each Akt isoform. While a MASO targeted to *akt-1* had no obvious effects, a MASO targeted to *akt-2* (the more highly expressed *akt* gene (Fig. 2B)) caused blastula stage arrest (Fig. 3A) followed by embryonic lethality. Remarkably, development of *akt-2* morphants was rescued by *akt-1* mRNA overexpression (supplementary material Fig. S3A), indicating

that the morphant phenotype is specifically caused by a deficit of Akt activity and that the two Akt isoforms are to some extent functionally redundant. Given this result and the fact that Akt-2 is the predominant isoform in early development, we chose to use the *akt-2* MASO for the following functional studies and did not further pursue Akt-1 knockdown.



In *akt-2* morphants, cell division arrested at the end of cleavage stage (60–120 cells, 10 hpf), as assessed by cell counts (Fig. 3B), a defect that was rescued by *akt-1* mRNA (supplementary material Fig. S3B). Overexpressing mRNA encoding a dominant negative mutant of Akt-1 (*akt-1-K186M*, containing a single base substitution that is known to abolish Akt kinase activity (Aoki et al., 1998)), but not mRNA encoding wild-type Akt-1, blocked cell division at the same stage (Fig. 3B), providing further evidence that the cell division defect produced by the *akt-2* MASO is attributable to its blockade of Akt function. This defect correlates with significantly reduced blastula stage incorporation of 5-ethynyl-2′-deoxyuridine (EdU) into nuclear DNA (Fig. 3C,D), indicating that loss of Akt function impairs transit into S phase. This conclusion is further substantiated by the fact that blastulae treated with the Akt inhibitor API-2 (Yang et al., 2004) also displayed significantly reduced EdU incorporation (Fig. 3E,F), as well as reduced cell numbers (Fig. 3G).

One way that Akt is known to promote cell proliferation is by phosphorylating and thereby inhibiting glycogen synthase kinase 3 (GSK-3; note that there is only one sea urchin homologue of the two vertebrate isoforms, GSK-3α and GSK-3β), which targets mitogenic proteins such as cyclin D and β-catenin for ubiquitin-mediated destruction (Holmes et al., 2008; Takahashi-Yanaga and Sasaguri, 2008). To determine if GSK-3 activity accounts for the loss of cell proliferation in Akt-inhibited embryos, we asked whether the negative effect of API-2 on cell proliferation could be countered by the GSK-3 inhibitor SB216763. This was indeed the case: embryos treated with both inhibitors had increased levels of late blastula stage EdU incorporation compared to embryos treated with API-2 alone, and normal cell numbers at 24 hpf (supplementary material Fig. S4A–C). However, SB216763 did not rescue cell division in either *akt-2* morphants or embryos overexpressing *akt-1-K186M* (supplementary material Fig. S4D–F), suggesting that GSK-3 is not the sole target of mitogenic Akt function. The reason for the difference between the results obtained with the Akt inhibitor and those obtained with the molecular perturbations is not known, although it may be that the molecular perturbations (knockdown and dominant-negative) affect a wider array of Akt functions than the inhibitor.

Fig. 3. Effects of perturbing Akt function on development and blastula stage cell proliferation. (**A**) Morphological effects of MASOs directed against *akt-1* and *akt-2*. Scale bar: 20 µm. (**B**) Cell numbers in control and *akt-2* morphants, and in embryos over-expressing wild type and kinase-dead (K186M) *akt-1* mRNA, from 10–15 hpf. (**C**) Confocal Projections of control and *akt-2* morphants labeled with EdU from 14–20 hpf. The EdU label (red) is incorporated into replicating DNA during the period of exposure; the DAPI (blue) indicates the total nuclear DNA. Scale bar: 20 µm. (**D**) Quantification of EdU labeling from the experiment shown in (B), showing the average number of nuclei per embryo displaying an EdU fluorescence intensity above a specific threshold (>25% of the DAPI signal), ± the SD. (**E**) Projections of fluorescent confocal images taken of untreated control embryos and embryos treated immediately after fertilization with the Akt inhibitor API-2 (25 µM) then labeled with EdU (red) for 2 hrs, beginning at 18 hrs post-fertilization; the DAPI (blue) indicates the total nuclear DNA. Scale bar: 20 µm. (**F**) Quantification of the results of an EdU labeling experiment, showing the number of nuclei displaying an EdU fluorescence intensity above a specific threshold (>25% of the DAPI signal), ± the SD. A total of 200 nuclei were counted for each sample, representing 2 control and 4 treated embryos. (**G**) Effects of API-2 treatment (5 µM) on total cell numbers at late blastula stage. Bars indicate average cell number per embryo from 10–15 embryos, ± the SD. Significance values calculated by *t*-test.

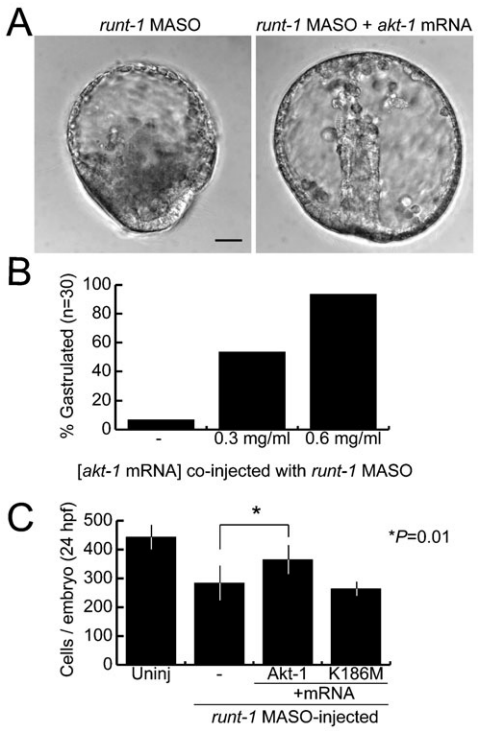


Fig. 4. Rescue of development in *runt-1* morphants with *akt-1* mRNA. (**A**) Two day *runt-1* morphant displaying gastrulation defective phenotype, and *runt-1* morphant co-injected with full-length *akt* mRNA at 0.3 mg/ml. Scale bar: 20 µm. (**B**) Quantification of gastrulation in runt-1 morphants co-injected with increasing [*akt* mRNA]. (**C**) Average cell numbers ± SD in 24 hr uninjected late blastula stage embryos, *runt-1* morphants, and *runt-1* morphants co-injected of *akt-1* and *akt-1-K186M* mRNA. Six to ten embryos were counted in each group. Significance value calculated by *t*-test.

Overexpression of akt-1 mRNA rescues development in runt-1 morphants

To determine if loss of Akt expression contributes to the defective development of *runt-1* morphants we asked whether the latter could be rescued by *akt* mRNA overexpression. Toward that end coinjected full-length *akt-1* mRNA into fertilized eggs along with the *runt-1* MASO. Remarkably, this rescues gastrulation in *runt-1* morphants in a dose-dependent manner (Fig. 4A,B), similar to the rescue obtained previously using *pkc1* mRNA overexpression (Dickey-Sims et al., 2005). Rescue of gastrulation was not achieved with equivalent concentrations of *akt-1-K186* mRNA (not shown), indicating that the rescue effect is mediated by Akt kinase activity. Blastula stage cell proliferation in *runt-1* morphants was also significantly increased by *akt-1* mRNA, but not by *akt-1-K186M* mRNA (which did not enhance the cell proliferation deficit in *runt-1* morphants; Fig. 4C). We conclude that Runx-dependent expression of *akt* contributes to blastula stage mitogenesis in the sea urchin.

Discussion

In sea urchins, as in many other animals, control of the cell cycle is turned over to the zygotic genome at blastula stage, following 6–7 maternally driven cleavage cycles. Given that these early cleavage cycles are autonomous and lack gap phases, whereas somatic cell proliferation depends on mitogenic intercellular signaling that controls the G1 to S and/or G2 to M phase transitions, it would be expected that the initial development of a somatic cell cycle in the embryo would hinge on the transcriptional activation of key mitogenic signaling molecules. Our previous studies suggested that the Runx transcription factor Runt-1 is critical for this aspect of development, being required for blastula stage cell division and expression of mitogenic signaling genes such as *pkc1*, *cyclinD*, and several *wnts*. As described in this report, by using a microarray to screen for genes mis-expressed in mid-blastula stage *runt-1* morphants, we identified *akt*, which encodes a serine/threonine kinase well known for its mitogenic and anti-apoptotic functions in mediating PI3-kinase signaling (Cantley, 2002), as another such gene. In *S. purpuratus*, *akt* is represented by two homologues, *akt-1* and *akt-2*, both of which are zygotically expressed contingent on *runt-1* expression, as shown by qRT-PCR (Fig. 2).

Our results indicate that Akt is a key mediator of mitogenic Runx function. Blocking Akt expression, by MASO-mediated knockdown of Akt-2 (the more highly expressed isoform), or function, by overexpression of a dominant negative (kinase-dead) mutant of Akt-1, impedes post-cleavage blastula stage cell division (Fig. 3B). Pharmacological inhibition of Akt also impairs blastula stage cell division (Fig. 3E–G). As expected based on the known mitogenic function of Akt, this involves impaired transition into S phase, as shown by EdU incorporation studies (Fig. 3C–F). Conversely, overexpression of *akt-1* mRNA rescues cell proliferation in *runt-1* morphants, which otherwise display reduced cell numbers at late blastula stage (Fig. 4C) (Robertson et al., 2008). The fact that overexpression of *akt-1 K186M* mRNA does not enhance the cell proliferation defect in blastula stage *runt-1* morphants (Fig. 4C) further suggests that *akt* activity mediates the mitogenic function of *runt-1* at that stage.

Previously we reported that development of *runt-1* morphants is rescued by overexpression of mRNA encoding the conventional protein kinase C (cPKC) PKC1 (Dickey-Sims et al., 2005), as well as by pharmacological inhibition of GSK-3, which resulted in stabilization of Runt-1 protein (Robertson et al., 2008). Those results, together with those presented here, suggest that Runx and GSK-3 are linked in a mutually antagonistic regulatory circuit (Fig. 5). Both Akt and cPKC are known to inhibit GSK-3 by phosphorylating conserved serine residues in the latter (Vilimek and Duronio, 2006), whereas canonical Wnt signaling inhibits GSK-3 by way of Disheveled (Cliffe et al., 2003). We have now shown that blastula stage expression of each of these GSK-3 antagonists is Runx-dependent, suggesting that a Runx promotes somatic cell proliferation by mobilizing a battery of genes that work in a self-reinforcing way to inhibit GSK-3 (Fig. 5). The observation that development of *runt-1* morphants can be rescued by overexpression of either Akt or PKC1, and also by pharmacological inhibition of GSK-3, is most parsimoniously explained by the observation that Runt-1 is stabilized by GSK-3 inhibition (Robertson et al., 2008). Thus, the rescue of *runt-1* morphants by both Akt and PKC1 may simply be due to restoration of functional Runt-1 protein levels in the morphants owing to decreased turnover. Further experiments are required to test this hypothesis.

Although not addressed in this study, Akt is well known for being anti-apoptotic, in addition to being pro-growth. The failure of *runt-1* morphants to gastrulate is caused by the widespread apoptosis that occurs in those embryos, as gastrulation can be rescued in *runt-1* morphants simply by inhibiting Caspase-3 (Dickey-Sims et al., 2005). *Akt-2* morphants arrest development at blastula stage, with a phenotype (Fig. 3A) that is at least

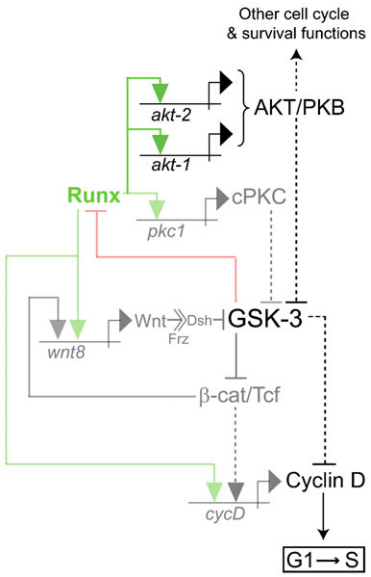


Fig. 5. A hypothetical regulatory circuit through which Runt-1 regulates cell proliferation in the mid-to-late blastula stage embryo. Gene names are italicized below the symbol for the corresponding gene. Protein products are capitalized. Positive *cis*-regulatory inputs are shown as arrows terminating on genes; negative (inhibitory) protein–protein regulatory interactions are shown as bars terminating on the named proteins; stimulatory effects are shown as arrows. Solid lines represent experimentally verified or well-established interactions/effects; dashed lines represent hypothetical interactions/effects. Interactions/effects not analyzed in this paper are shown in the background. Evidence and supporting references for the other interactions are given in Robertson et al. (Robertson et al., 2008).

superficially similar to the apoptotic phenotype displayed by both *runt-1* morphants (Fig. 1B, Fig. 4A) and *pkc1* morphants (Dickey-Sims et al., 2005). Thus, it seems likely that in addition to contributing to the mitogenic function of Runx, Akt contributes to its anti-apoptotic function previously attributed to PKC1. However, since the rescue effects of both Akt and PKC1 may simply be due to stabilization of Runt-1 protein via GSK-3 inhibition, it cannot be concluded from the work done so far that either Akt or PKC1 (or a combination of both) directly mediates the anti-apoptotic function of Runt-1. Further work is required to elucidate the specific developmental roles of each of these players, which undoubtedly involve complex networks of interactions that defy simple linear models of cause and effect.

Materials and Methods

Sea urchins, embryo culture, microinjection, molecular reagents and inhibitor treatments

Adult *S. purpuratus* were obtained from Charles Hollahan (Santa Barbara Marine Biologicals) or Pat Leahy (Pt. Loma Marine Invertebrate Lab), and induced to spawn by vigorous shaking. Eggs were dejellied by a brief wash in pH 4.8 seawater, and fertilized with a dilute sperm suspension in 1 mM para-aminobenzoic acid (PABA) in filtered seawater. Microinjection was carried out essentially as described by Cheers and Ettensohn, using injection solutions containing 120 mM KCl together with MASOs at a concentration of 100–500 µM and/or mRNA at a concentration of 300–500 mg/ml (Cheers and Ettensohn, 2004). The splice-blocking anti-SpRunt-1 MASO (m5) was described previously (Coffman et al., 2004). The sequences of the translation-blocking Akt MASOs are CCGAGACCGACATCGTCGTCGTCAT (Akt-1), and GCTTCCGACAT-TGTTGTTGTTATCA (Akt-2). Akt-1 mRNA was synthesized from *not-1*-linearized plasmid encoding Akt-1 obtained from our full-length arrayed plasmid/EST library in pCMVSport6.1 (NCBI acc. no. CX555355). The same plasmid served as template to make the kinase-dead Akt-1-K186M point mutant using the QuickChange II method (Stratagene) with primers complementary to the Akt-1 sequence (highlighted in supplementary material Fig. S1). The resulting plasmid was sequenced to verify the point mutation and linearized with *not-1* for mRNA synthesis. mRNA for microinjection was synthesized *in vitro* from the linearized plasmids using the Sp6 mMessage mMachine (Ambion). Embryos were developed in filtered seawater (FSW) at 15°C. The kinase inhibitors API-2 (Tocris, 5–25 µM final concentration) and SB216763 (Tocris, 1 µM) were added to the FSW culture medium immediately after fertilization.

Microarray design and analysis

The EST database for the sea urchin *Strongylocentrotus purpuratus* was interrogated to identify sequences that are expressed in the late blastula (24 hr) embryo, a stage that is expected to express all genes relevant to *runt-1* function in early development. The EST sequences were assembled with all other available ESTs and clustered into a 24 hr embryo 'uni-gene' set. From the initial set of 36,230 blastula stage ESTs, a non-redundant set of ~6,800 annotated sequences was obtained for microarray analysis. This set included 803 developmentally significant genes from the sea urchin genome from the following annotation categories: transcription factors, cell-signaling, apoptosis, and cell cycle/growth/DNA damage repair. The uni-gene set of expressed sequences was used to design ~13,600 60mer oligonucleotides ('oligos', two per expressed sequence) for a custom 8×15 k Agilent microarray purchased from Agilent, Inc.

Total RNA was extracted from 4 biological replicates of 18 hr blastula stage morphants (~5,000 embryos per replicate) and similar quantities of untreated control embryos using the RNeasy Kit (Qiagen, Valencia, CA). The RNA was provided to the microarray core Facility of the Cornell University Veterinary School (Ithaca, NY) where the labeling was performed using an Ambion MessageAmp kit (Applied Biosystems/Ambion, Austin, TX) optimized to produce maximum length labeled aRNA from an oligo dT directed polymerase start position.

The microarray images were processed at the Cornell University Life Sciences Core Laboratory Center using Agilent Feature Extraction Software. The results were exported to text as tsv files and provided as such. Computing was performed in Microsoft Excel. First, a scalar normalization factor was calculated and applied to the fluorescence values of dye-swap partner data sets prior to further calculation. No normalization was performed among biological replicates, each sample-control set being treated independently from the others. For 8 data sets (the dye-swap labeled targets of 4 biological replicates), filters were developed within the prepared Excel spreadsheets to find consistently up- or downregulated genes. Briefly, for each data set the difference measure of the ratios of sample fluorescence to that of the corresponding control were calculated, ranked and all features within the range of 0.5 to 1.5 were removed. Fluorescent ratios of 1.5 or greater were compared among data sets and so for ratios 0.5 or less in order to find the overlap of features consistently regulated among the replicates. Panglos, the online Venn diagram tool (http://www.pangloss.com/seidel/Protocols/venn.cgi), was used to identify the intersection of features common to each of the resulting filtered lists. The final lists of consistently affected genes were manually curated to include genes that were found to be near the margins of the filter in only 1 replicate while passing filter in others. The full data obtained from the results of the microarray analysis have been deposited in the NCBI Gene Omnibus database (GEO), under accession number GSE19751.

Quantitative reverse transcription coupled polymerase chain reaction (qRT-PCR)

Total RNA was extracted from blastula stage *runt-1* morphants and control embryos as described above, and subjected to qRT-PCR analysis using primer pairs complementary to sequences in Akt-1 and Akt-2 that are highlighted in supplementary material Fig. S1, and ubiquitin primers (for normalization) as described previously (Robertson et al., 2008).

Analysis of cell proliferation

Cell proliferation was detected using the Click-IT EdU labeling kit (Invitrogen) following the manufacturer's instructions, and by direct counts of nuclei in squashed embryos fluorescently labeled with Vybrant DyeCycle Green (Invitrogen) as previously described (Robertson et al., 2008).

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Author Contributions

J.A.C. and A.J.R. conceived and designed the experiments; A.J.R. analyzed the microarray data; A.J.R. and J.A.C. performed the qPCR; J.A.C. performed the microinjections and some of the

imaging and cell counts; A.C., K.R., and S.J. performed imaging and image analysis of cell proliferation.

Competing Interests

The authors have no competing interests to declare.

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