Development 135, 3185-3190 (2008) doi:10.1242/dev.026443

GATA transcription factors integrate Wnt signalling during heart development

Boni A. Afouda[1], Jennifer Martin[1], Fei Liu[1,*], Aldo Ciau-Uitz[2], Roger Patient[2] and Stefan Hoppler[1,†]

Cardiogenesis is inhibited by canonical Wnt/β-catenin signalling and stimulated by non-canonical Wnt11/JNK signalling, but how these two signalling pathways crosstalk is currently unknown. Here, we show that Wnt/β-catenin signalling restricts cardiogenesis via inhibition of GATA gene expression, as experimentally reinstating GATA function overrides β-catenin-mediated inhibition and restores cardiogenesis. Furthermore, we show that GATA transcription factors in turn directly regulate Wnt11 gene expression, and that Wnt11 is required to a significant degree for mediating the cardiogenesis-promoting function of GATA transcription factors. These results demonstrate that GATA factors occupy a central position between canonical and non-canonical Wnt signalling in regulating heart muscle formation.

KEY WORDS: Cardiomyogenesis, GATA, Heart, Wnt, *Xenopus*

INTRODUCTION

The GATA family of transcription factors plays a pivotal role near the top of the cascade of events that lead to heart muscle differentiation (Brewer and Pizzey, 2006; Peterkin et al., 2005; Peterkin et al., 2007). Vertebrate GATA4, GATA5 and GATA6 are expressed in early cardiogenic tissue (Molkentin, 2000; Patient and McGhee, 2002), and regulate cardiomyogenesis, in a partially redundant manner (Holtzinger and Evans, 2007; Kuo et al., 1997; Molkentin et al., 1997; Peterkin et al., 2007). However, the precise roles and molecular targets of GATA transcription factors in vertebrate heart formation are just beginning to be discovered.

Extracellular signals that regulate cardiogenesis have been primarily identified by their ability to induce cardiac differentiation in non-cardiac mesoderm. Among these are Wnt signals, such as Wnt11 (Pandur et al., 2002), but also secreted Wnt antagonists, such as Dickkopf-1 and Crescent (David et al., 2008; Marvin et al., 2001; Schneider and Mercola, 2001). Wnt antagonists and Wnt signals were both found to promote heart development because cardiogenesis requires both repression of canonical Wnt/β-catenin signalling, which would otherwise inhibit cardiogenesis (Marvin et al., 2001; Schneider and Mercola, 2001), and activation of non-canonical Wnt11/JNK signalling, which promotes cardiogenesis (Eisenberg and Eisenberg, 1999; Pandur et al., 2002; Terami et al., 2004). These findings raise the important issue of how these two Wnt signalling pathways interact to control cardiogenesis.

Because of their prominent roles in cardiogenesis, we investigated the relationship between GATA transcription factors and the two Wnt signalling pathways.

[1]Institute of Medical Sciences, Cell and Developmental Biology Research Programme, School of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK. [2]MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK.

*Present address: Institute of Regenerative Medicine, A&M System Health Science Center, 5701 Airport Road, Module C, Temple, Texas 76502, USA
†Author for correspondence (e-mail: s.p.hoppler@abdn.ac.uk)

Accepted 29 July 2008

MATERIALS AND METHODS

Expression constructs and morpholino

GATA4GR and GATA6GR, activin (Afouda et al., 2005) and Dickkopf-1 (Semenov et al., 2001) mRNA expression constructs have been previously described. β-cateninGR is a hormone-inducible β-catenin. Wnt11 (Pandur et al., 2002), GATA4 (Afouda et al., 2005) and GATA6 (Peterkin et al., 2003) morpholinos have been previously characterised.

Embryos and explants culture

For animal cap assays, *Xenopus* embryos were injected at the one-cell stage into the animal pole. Embryos were injected at the four-cell stage into ventral blastomeres for the ventral marginal zone (VMZ) explant experiments or into dorsal blastomeres for the dorsal marginal zone (DMZ) explant experiments and the whole embryo analysis. mRNA and morpholino (MO) were injected in sterile autoclaved water (10 nl). The total amount of MO injected was 5 ng (Wnt11 MO), 10 ng (GATA6 MO) and 20 ng (GATA4 MO) per embryo. Animal cap explants were dissected at stage 8 and cultured in 0.6×MMR as described previously (Afouda et al., 2005). Cycloheximide treatment was carried out as previously described (Tada et al., 1997). DMZ or VMZ explants were dissected at stage 10, when the prospective dorsal side is clearly identifiable, and cultured in 0.6×MMR. Explants were cultured until the appropriate control stage before processing for analysis of RNA expression. Sets of experiments were repeated at least twice in order to confirm results presented here. Cardiomyocyte differentiation of animal cap and DMZ explants was analysed at phenotypical level at control stage 45 and expressed as a percentage of rhythmically beating explants compared with non-beating, but otherwise healthy, explants.

RNA expression analysis by RT-PCR

Total RNA extraction and the reverse transcription (RT) reaction were performed as previously described (Afouda et al., 2005). PCR reactions on cDNA were calibrated to be strictly in the linear range (with a linearity control series for each figure) and compared with ODC expression control. Primer sequences and PCR conditions are available from the authors upon request.

RESULTS AND DISCUSSION

The cardiogenesis-specific functions of Wnt signalling and GATA transcription factors are difficult to study in whole embryos because both Wnt signalling and GATA transcription factors are also important regulators at other developmental stages and in other embryonic tissues (Afouda et al., 2005; Liu et al., 2005; Tada and Smith, 2000). We have therefore adapted reliable

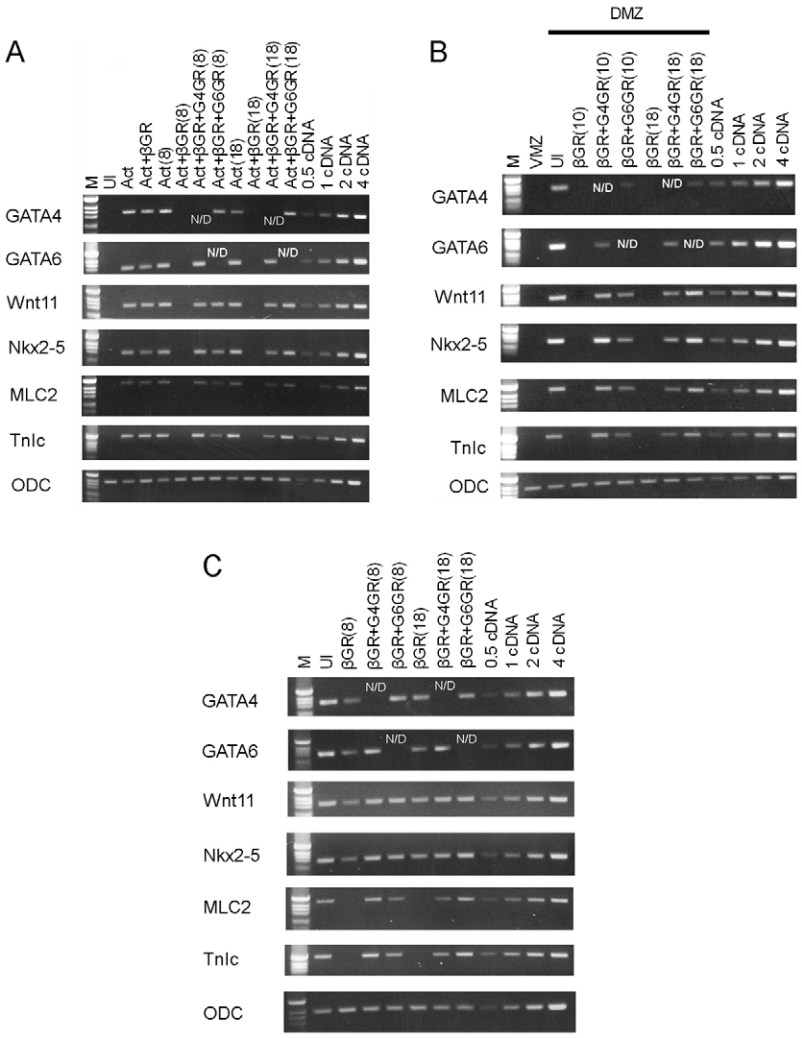


Fig. 1. Wnt/β-catenin-mediated inhibition of cardiogenesis in *Xenopus* is via GATA4/6 function. Gene expression analysis at stage 32 by RT-PCR of animal cap explants, injected with RNA encoding Activin (Act, 50 fg) to induce cardiogenesis (A), dorsal marginal zone (DMZ) explants (B) and whole embryos (C), which were injected with β-cateninGR (βGR, 50 pg), GATA4GR (G4GR, 200 pg) or GATA6GR (G6GR, 200 pg), as indicated, and were cultured with dexamethasone from stages 8, 10 or 18 where indicated. Activation of β-catenin abolishes activin-induced (**A**) and endogenous (**B**) expression of GATA4 and GATA6, as well as cardiogenesis, as monitored by marker gene expression. (**C**) In whole embryo analysis, activation of β-catenin abolishes heart muscle-specific differentiation markers (MLC2, TnIc), but causes only a relatively weak reduction of markers that are not exclusively heart specific (GATA4, GATA6, Wnt11, Nkx2-5). However, β-catenin-mediated inhibition of cardiogenic marker gene expression is rescued by concomitant activation of GATA4GR or GATA6GR in all three assays. N/D, not determined; VMZ, ventral marginal zone explant.

Xenopus animal cap and cardiac mesoderm explant assays (Ariizumi et al., 2003; Latinkic et al., 2003; Schneider and Mercola, 2001), which allow us to examine in isolation the regulatory mechanisms by which Wnt/β-catenin and Wnt11/JNK signalling, and GATA transcription factors, interact to regulate cardiogenesis.

GATA factors are the relevant targets for inhibition by canonical Wnt signalling

Activin mRNA-injected *Xenopus* animal cap explants initiate cardiogenesis, as monitored by cardiogenic marker gene expression (GATA4, GATA6, Wnt11, Nkx2-5, MLC2 and TnIc; Fig. 1A) and by differentiation into functional heart muscle (Ariizumi et al., 2003). In order to confirm that Wnt/β-catenin signalling can inhibit development of cardiogenic mesoderm, we activated the pathway in a temporally controlled manner by co-injecting β-cateninGR mRNA (hormone-inducible version of β-catenin) and activating it with dexamethasone. Strikingly, the activin-induced expression of GATA transcription factors and other cardiogenic markers was abolished when β-cateninGR was activated at relatively early stages, when cardiogenic mesoderm is being induced (stage 8), but also later during subsequent heart development (stage 18) (Fig. 1A).

Because of the observed inhibition of endogenous GATA expression by β-catenin signalling and the prominent cardiogenesis-promoting role of GATA factors (Latinkic et al., 2003; Peterkin et al., 2003; Peterkin et al., 2007), we tested whether GATA genes are the relevant targets of Wnt/β-catenin signalling in cardiogenesis. We reinstated GATA function with hormone-inducible GATA proteins (Afouda et al., 2005) that are activated at the same time as co-injected β-cateninGR was causing reduced expression of the endogenous GATA genes. The inhibition of cardiogenic marker gene expression by β-cateninGR was indeed rescued by activating GATA4GR and GATA6GR (Fig. 1A). This result suggests a regulatory pathway in which Wnt/β-catenin signalling restricts cardiogenesis by inhibiting GATA gene expression.

In order to substantiate our findings, we conducted similar experiments using dorsal marginal zone (DMZ) explants (Fig. 1B) and analysis in whole embryos (Fig. 1C). DMZ explants differentiate into heart tissue in the absence of added factors (Foley and Mercola, 2005; Schneider and Mercola, 2001). Activation of β-cateninGR either at stage 10 or 18 strongly reduced the expression of GATA genes and other heart development markers, yet their expression was restored by reinstated GATA4 or GATA6 activity (Fig. 1B). As expected, in the whole embryo analysis the β-catenin-mediated reduction of cardiogenic gene expression is only clearly

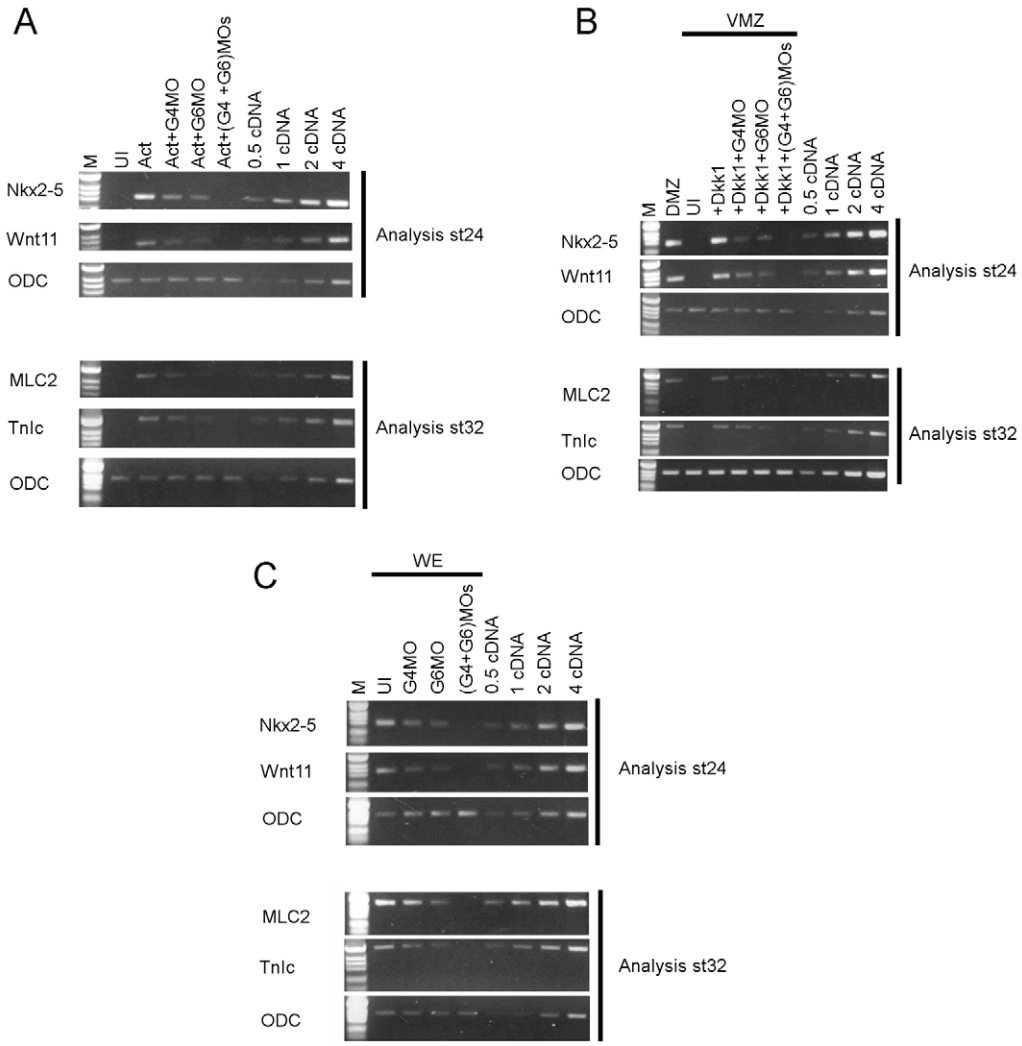


Fig. 2. GATA4 and GATA6 are required for Wnt11 expression and heart muscle differentiation in *Xenopus*. RT-PCR analysis of Nkx2-5, Wnt11, MLC2 and TnIc gene expression in embryonic explants and whole embryos at control stages 24 and 32, as indicated. (**A**) Animal cap explants injected with RNA encoding Activin (Act, 50 fg) to induce cardiogenesis and in combination with morpholinos targeting GATA4 or GATA6 (G4MO, G6MO), as indicated. (**B**) Ventral marginal zone (VMZ) explants injected with Dickkopf-1 mRNA (Dkk-1, 800 pg) to induce ectopic cardiogenesis and together with G4MO, or G6MO as indicated. (**C**) Analysis of gene expression in whole embryos injected with G4MO or G6MO as indicated. Note that morpholino-mediated inhibition of either GATA4 or GATA6 reduces Wnt11 and cardiogenic marker gene expression, but that inhibition of both GATA4 and 6 abolishes them in all three assays.

detectable in the strictly heart muscle-specific genes MLC2 and TnIc; nevertheless, their expression is restored by activated GATA function (Fig. 1C, see Fig. S1 in the supplementary material). Our results demonstrate that GATA transcription factors are sufficient to overcome the negative regulation of cardiogenesis by Wnt/β-catenin signalling and therefore suggest that GATA transcription factors act downstream of Wnt/β-catenin signalling in cardiomyogenesis.

GATA function is required for Wnt11 expression and cardiomyogenesis

The requirement for GATA function in cardiogenesis is complicated by extensive redundancy between different GATA genes (Holtzinger and Evans, 2007; Peterkin et al., 2007). In order to test whether GATA function is required for Wnt11 expression and cardiomyogenesis, we used morpholinos to inhibit GATA4 and GATA6 expression. We first used activin-injected animal caps as before to induce cardiogenic marker gene expression (Fig. 2A) but also VMZ explants injected with Dkk-1 mRNA to induce ectopic cardiogenesis (Fig. 2B) (Foley and Mercola, 2005; Marvin et al., 2001; Schneider and Mercola, 2001) and intact embryos (Fig. 2C). In all three assays, we found that inhibition of either GATA4 or GATA6 alone caused some reduction in gene expression of Wnt11 and other cardiogenic markers, but a much stronger reduction when both were inhibited, confirming that Wnt11 gene expression and heart development are dependent on normal GATA function.

Nkx2-5 and Wnt11 are direct targets for GATA4 and GATA6

The pro-cardiogenic activity of GATA transcription factors in overexpression experiments (Gove et al., 1997; Latinkic et al., 2003; Reiter et al., 1999) is shared with Wnt11 (Eisenberg and Eisenberg, 1999; Pandur et al., 2002; Terami et al., 2004), which is co-expressed with GATA factors during early cardiogenesis (Ku and Melton, 1993) (see Fig. S3 in the supplementary material). We therefore investigated whether Wnt11 is a direct target of GATA transcription factors in cardiomyogenesis. We activated GATAGR proteins with dexamethasone in the presence of protein synthesis inhibitors that prevent indirect gene induction, and assayed for Wnt11 and Nkx2-5 expression. Controls without dexamethasone showed no or low Nkx2-5 gene expression. With dexamethasone, Nkx2-5 expression was induced during early stages (Fig. 3A) and at later stages (Fig. 3B), even in the presence of cycloheximide, confirming Nkx2-5 as a direct target of GATA regulation (Brewer et al., 2005; Searcy et al., 1998). Importantly, our experiments show for the first time that Wnt11 gene expression is also directly

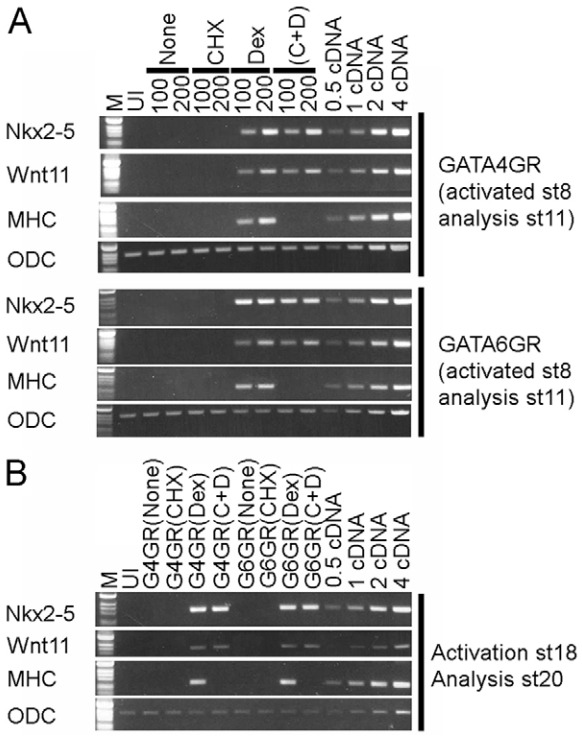


Fig. 3. Wnt11 and Nkx2-5, but not MHC are direct targets of regulation by GATA4 and GATA6. RT-PCR analysis of Nkx2-5, Wnt11 and MHC gene expression in *Xenopus* animal cap explants that were injected with GATA4GR or GATA6GR RNA and cultured in the presence or absence of cycloheximide (CHX) and dexamethasone (Dex), as indicated. In A, 100 pg or 200 pg GATA4GR or GATA6GR mRNA were injected, as indicated; in B, 100 pg. (**A**) CHX followed by Dex treatment from stage 8 and analysis of gene expression at control stage 11. (**B**) CHX followed by Dex treatment from control stage 18 and analysis at stage 20. Activated GATA transcription factors induce Nkx2-5 and Wnt11, but not MHC gene expression in the presence of the protein synthesis inhibitor (CHX) at different stages, thereby identifying them as direct target genes of GATA transcription factors.

regulated by GATA transcription factors (Fig. 3A,B), as Wnt11 gene expression was induced by dexamethasone-activated GATA4GR or GATA6GR, even when protein synthesis was inhibited by cycloheximide, unlike the negative control myosin heavy chain (MHCα).

Wnt11 function is required downstream of GATA4 and GATA6 in cardiomyogenesis

GATA4 can drive full cardiomyogenesis in *Xenopus* animal pole explants, leading to cardiogenic gene expression and differentiation into beating cardiomyocytes (Latinkic et al., 2003). Furthermore, Wnt11 activation of a JNK-dependent pathway is required for cardiogenesis in *Xenopus*, as well as in cell culture models (Pandur et al., 2002). As we had found that expression of Wnt11 requires GATA function and that it was even among direct targets of regulation by GATA transcription factors, we investigated the requirement for Wnt11 downstream of GATA function using a Wnt11 morpholino (11MO) (Pandur et al., 2002) together with GATAGR mRNAs.

We found that either GATA4GR or GATA6GR were able to initiate cardiomyogenic gene expression (MLC2, TnIc) in animal cap explants (Fig. 4A), but that only GATA4GR was able to induce beating cardiomyocytes (Fig. 4B,D). We had earlier noticed subtle but consistent differences between GATA4GR and GATA6GR activities (Fig. 1, see Fig. S2 in the supplementary material), indicating that GATA4GR is a slightly stronger inducer of cardiogenic marker gene expression when activated at earlier stages, whereas GATA6GR the relatively stronger inducer when activated later. Our investigation does not address whether this is simply due to technical differences between the GATA4GR and the GATA6GR constructs or whether it reflects functional differences between the endogenous genes (Nemer, 2008; Peterkin et al., 2005; Peterkin et al., 2007; Xin et al., 2006; Zhao et al., 2008), but we think it is related to their differing abilities to induce beating cardiomyocytes (Charron et al., 2001; Latinkic et al., 2003). Nevertheless, morpholino-mediated inhibition of Wnt11 caused clear reduction of cardiomyogenic gene expression (Fig. 4A,C) and reduced differentiation into beating cardiomyocytes (Fig. 4D). These results show that Wnt11 is required to a significant degree for mediating the cardiogenesis-promoting activity of GATA factors.

To confirm that the need for Wnt11 function is not confined to artificially induced cardiogenesis, we tested whether Wnt11 function is also required for cardiomyogenic gene expression and cardiomyocyte differentiation in DMZ explants and in whole embryos. We found indeed that morpholino-mediated inhibition of Wnt11 causes reduced MLC2 and TnIc expression in DMZ explants and in whole embryos (Fig. 4E) and that it reduces the number of beating DMZ explants and embryos with a detectable heart beat (Fig. 4F). Our results therefore argue that Wnt11 is a key effector of cardiogenesis downstream of GATA transcription factors.

GATA factors link canonical and non-canonical Wnt signalling in cardiogenesis

Our results suggest a regulatory pathway controlling cardiomyogenesis whereby GATA transcription factors link canonical and non-canonical Wnt signalling. We have confirmed that Wnt/β-catenin signalling inhibits cardiogenesis (Cohen et al., 2008; Eisenberg and Eisenberg, 2006; Naito et al., 2006; Tzahor, 2007; Ueno et al., 2007), that GATA4/6 promote cardiomyogenesis (Latinkic et al., 2003; Peterkin et al., 2003; Peterkin et al., 2007) and that Wnt11 is required for cardiomyogenesis (Pandur et al., 2002). We also show for the first time that GATA4/6 can overrule Wnt/β-catenin-mediated inhibition of cardiogenesis, that GATA4/6 directly induces Wnt11 expression and that GATA4/6 promotes myocardial differentiation largely via Wnt11. These findings establish a hierarchy of regulation with canonical Wnt/β-catenin signalling restraining GATA gene expression, which otherwise induces non-canonical Wnt11/JNK signalling to promote subsequent aspects of heart muscle differentiation.

Our results do not necessarily argue for a strictly linear pathway. We find that repression of GATA gene expression by activated Wnt/β-catenin signalling is often not complete, which is likely to reflect additional regulation of GATA gene expression, for example by Nkx transcription factors (Molkentin et al., 2000) and by Wnt11 (Pandur et al., 2002), which may represent reinforcing regulatory loops operating together with the regulatory hierarchical pathway we describe. Furthermore, Wnt11 is not the only cardiogenesis-promoting factor induced by activated GATA; Nkx2-5, a key regulator of heart development (Brewer et al., 2005; Cripps and Olson, 2002), is also induced, which may suggest an alternative pathway downstream of GATA4/6. Our results provide further evidence for this notion in the observed incomplete abolition of cardiogenic gene expression and only reduced differentiation into beating cardiomyocytes when Wnt11

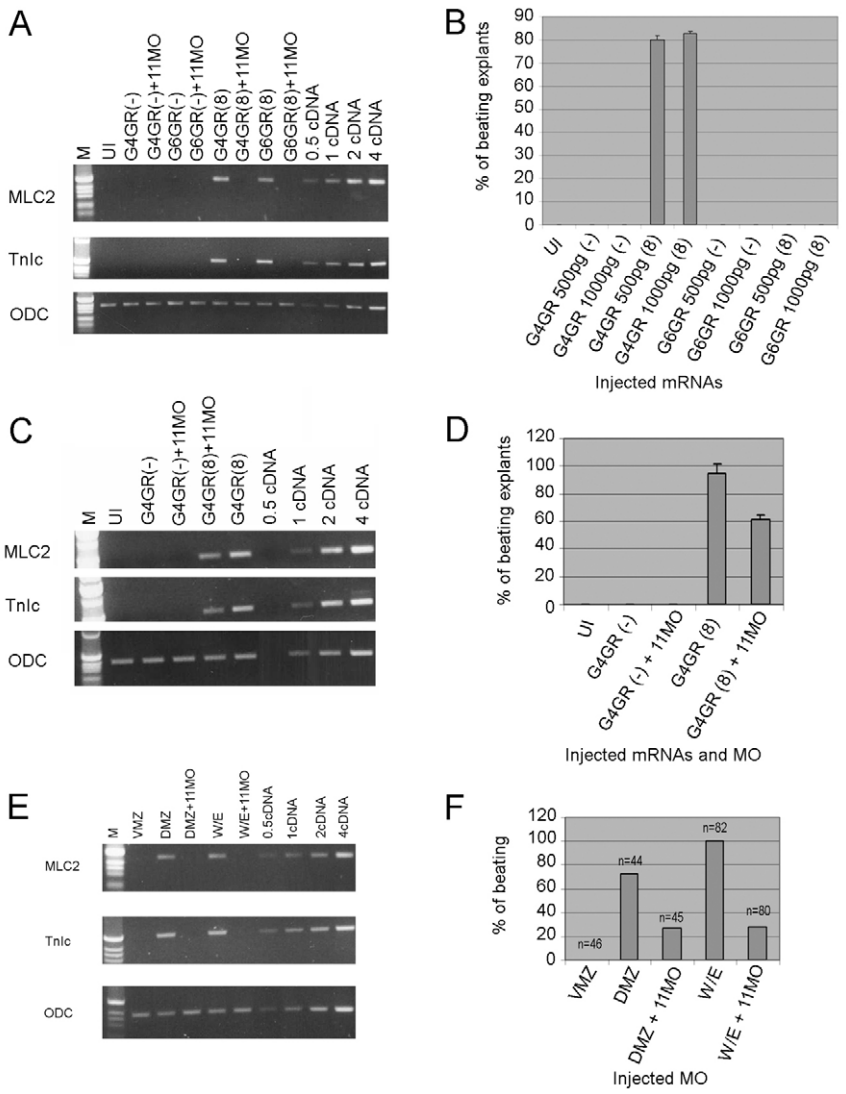


Fig. 4. Wnt11 is required for induction of cardiomyogenesis by GATA4 and GATA6. RT-PCR analysis of cardiomyogenic gene expression (MLC2, TnIc) at control stage 32 (A,C,E) and analysis of cardiomyocyte differentiation (detectable rhythmic beating) at control stage 45 (B,D,F). (**A**) *Xenopus* animal cap explants that were injected with GATA4GR or GATA6GR mRNA (G4GR, G6GR, 100 pg), alone or in combination with Wnt11 morpholino (11MO) as indicated, and cultured in the absence or in the presence of dexamethasone from stage 8. Induction of cardiomyogenic gene expression by G4GR and G6GR is abolished by inhibition of Wnt11. (**B**) Numerical analysis of spontaneous rhythmic beating in animal cap explants injected with G4GR or G6GR mRNA (500 pg or 1000 pg and activated with dexamethasone at stage 8, as indicated). Only G4GR mRNA is capable of inducing rhythmic beating in animal cap explants. (**C**) Analysis of cardiomyogenic gene expression in sibling animal cap explants of those used for cardiomyocyte differentiation in D, injected with G4GR (500 pg, activated at stage 8) with 11MO as indicated. (**D**) Numerical analysis of spontaneous rhythmic beating in sibling animal cap explants. Under these conditions (G4GR mRNA, 500 pg), inhibition of Wnt11 reduces, but does not abolish, cardiomyogenic gene expression, consistent with reduced cardiomyocyte differentiation in explants. (**E**) Cardiomyogenic gene expression in sibling dorsal marginal zone explants (DMZ) or in sibling whole embryos (W/E) of those used for phenotypic analysis in F, which were injected with 11MO as indicated. (**F**) Numerical analysis of rhythmic beating in DMZ explants and whole embryos, as in E. Morpholino-mediated inhibition of Wnt11 causes strong but not complete reduction of cardiomyogenic gene expression, consistent with the decreased cardiomyogenesis in both DMZ explants and whole embryos. In B,D, the results of three different experiments are combined but in F only the result of one experiment is presented with *n* representing the number of explants or embryos scored. VMZ, ventral marginal zone.

is inhibited, which is also consistent with the relatively mild heart phenotypes observed in the zebrafish silberblick (wnt11) mutation (Matsui et al., 2005) and the mouse Wnt11 knockout (Majumdar et al., 2003).

We thank Professor Makoto Asashima (University of Tokyo) for primer sequences and PCR conditions, Dr Danielle Lavery for discussions, and Yvonne Turnbull for technical assistance. This work was funded by The Wellcome Trust (071101/Z/03/Z), British Heart Foundation (PG/07/043) and the Royal Society.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/19/3185/DC1

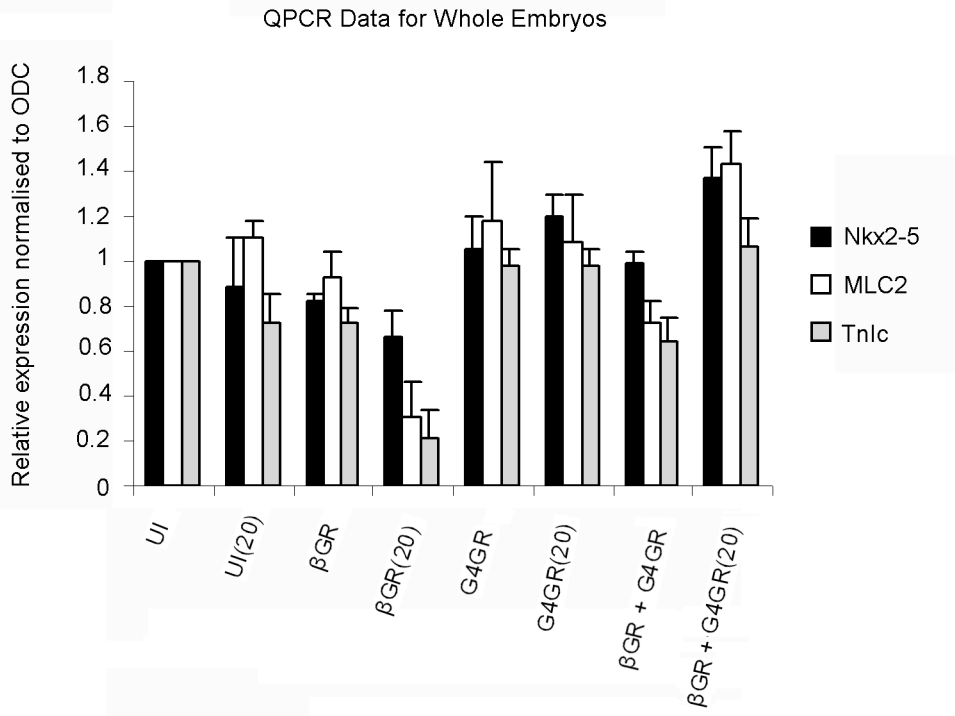
References

Afouda, B. A., Ciau-Uitz, A. and Patient, R. (2005). GATA4, 5 and 6 mediate TGFbeta maintenance of endodermal gene expression in Xenopus embryos. *Development* **132**, 763-774.

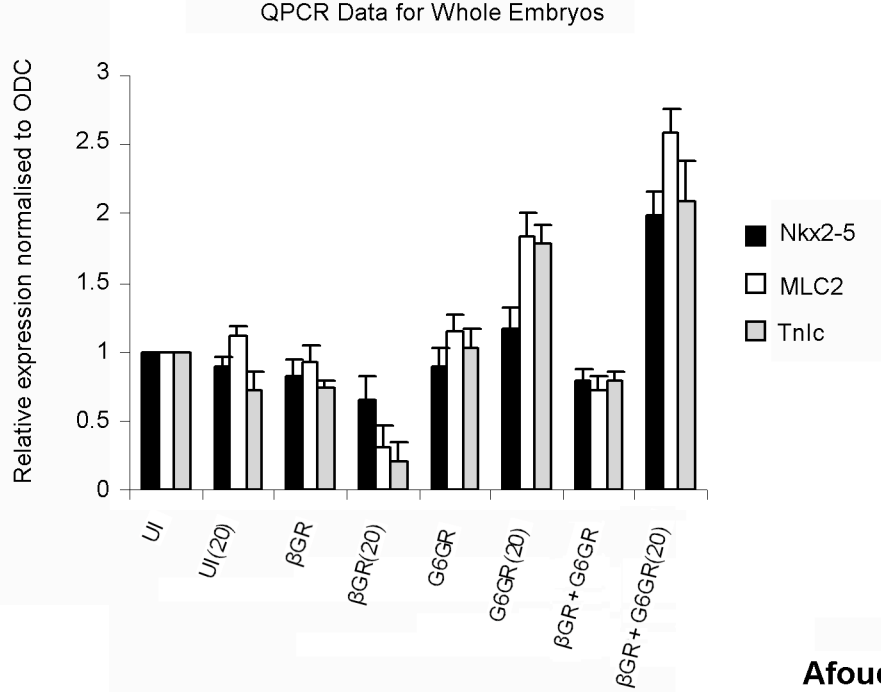
Ariizumi, T., Kinoshita, M., Yokota, C., Takano, K., Fukuda, K., Moriyama, N., Malacinski, G. M. and Asashima, M. (2003). Amphibian in vitro heart

induction: a simple and reliable model for the study of vertebrate cardiac development. *Int. J. Dev. Biol.* **47**, 405-410.
Brewer, A. and Pizzey, J. (2006). GATA factors in vertebrate heart development and disease. *Expert Rev. Mol. Med.* **8**, 1-20.
Brewer, A. C., Alexandrovich, A., Mjaatvedt, C. H., Shah, A. M., Patient, R. K. and Pizzey, J. A. (2005). GATA factors lie upstream of Nkx 2.5 in the transcriptional regulatory cascade that effects cardiogenesis. *Stem Cells Dev.* **14**, 425-439.
Charron, F., Tsimiklis, G., Arcand, M., Robitaille, L., Liang, Q., Molkentin, J. D., Meloche, S. and Nemer, M. (2001). Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev.* **15**, 2702-2719.
Cohen, E. D., Tian, Y. and Morrisey, E. E. (2008). Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. *Development* **135**, 789-798.
Cripps, R. M. and Olson, E. N. (2002). Control of cardiac development by an evolutionarily conserved transcriptional network. *Dev. Biol.* **246**, 14-28.
David, R., Brenner, C., Stieber, J., Schwarz, F., Brunner, S., Vollmer, M., Mentele, E., Muller-Hocker, J., Kitajima, S., Lickert, H. et al. (2008). MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. *Nat. Cell Biol.* **10**, 338-345.
Eisenberg, C. A. and Eisenberg, L. M. (1999). WNT11 promotes cardiac tissue formation of early mesoderm. *Dev. Dyn.* **216**, 45-58.
Eisenberg, L. M. and Eisenberg, C. A. (2006). Wnt signal transduction and the formation of the myocardium. *Dev. Biol.* **293**, 305-315.
Foley, A. C. and Mercola, M. (2005). Heart induction by Wnt antagonists depends on the homeodomain transcription factor Hex. *Genes Dev.* **19**, 387-396.
Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A. and Patient, R. (1997). Over-expression of GATA-6 in Xenopus embryos blocks differentiation of heart precursors. *EMBO J.* **16**, 355-368.
Holtzinger, A. and Evans, T. (2007). Gata5 and Gata6 are functionally redundant in zebrafish for specification of cardiomyocytes. *Dev. Biol.* **312**, 613-622.
Ku, M. and Melton, D. A. (1993). Xwnt-11: a maternally expressed Xenopus wnt gene. *Development* **119**, 1161-1173.
Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.* **11**, 1048-1060.
Latinkic, B. V., Kotecha, S. and Mohun, T. J. (2003). Induction of cardiomyocytes by GATA4 in Xenopus ectodermal explants. *Development* **130**, 3865-3876.
Liu, F., van den Broek, O., Destree, O. and Hoppler, S. (2005). Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/β-catenin signalling in mesoderm development. *Development* **132**, 5375-5385.
Majumdar, A., Vainio, S., Kispert, A., McMahon, J. and McMahon, A. P. (2003). Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* **130**, 3175-3185.
Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M. and Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev.* **15**, 316-327.
Matsui, T., Raya, A., Kawakami, Y., Callol-Massot, C., Capdevila, J., Rodriguez-Esteban, C. and Izpisua Belmonte, J. C. (2005). Noncanonical Wnt signaling regulates midline convergence of organ primordia during zebrafish development. *Genes Dev.* **19**, 164-175.
Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J. Biol. Chem.* **275**, 38949-38952.
Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* **11**, 1061-1072.
Molkentin, J. D., Antos, C., Mercer, B., Taigen, T., Miano, J. M. and Olson, E. N. (2000). Direct activation of a GATA6 cardiac enhancer by Nkx2.5: evidence for a reinforcing regulatory network of Nkx2.5 and GATA transcription factors in the developing heart. *Dev. Biol.* **217**, 301-309.
Naito, A. T., Shiojima, I., Akazawa, H., Hidaka, K., Morisaki, T., Kikuchi, A. and Komuro, I. (2006). Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc. Natl. Acad. Sci. USA* **103**, 19812-19817.
Nemer, M. (2008). Genetic insights into normal and abnormal heart development. *Cardiovasc. Pathol.* **17**, 48-54.
Pandur, P., Lasche, M., Eisenberg, L. M. and Kuhl, M. (2002). Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* **418**, 636-641.
Patient, R. K. and McGhee, J. D. (2002). The GATA family (vertebrates and invertebrates). *Curr. Opin. Genet. Dev.* **12**, 416-422.
Peterkin, T., Gibson, A. and Patient, R. (2003). GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. *EMBO J.* **22**, 4260-4273.
Peterkin, T., Gibson, A., Loose, M. and Patient, R. (2005). The roles of GATA-4, -5 and -6 in vertebrate heart development. *Semin. Cell Dev. Biol.* **16**, 83-94.
Peterkin, T., Gibson, A. and Patient, R. (2007). Redundancy and evolution of GATA factor requirements in development of the myocardium. *Dev. Biol.* **311**, 623-635.
Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13**, 2983-2995.
Schneider, V. A. and Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in Xenopus laevis. *Genes Dev.* **15**, 304-315.
Searcy, R. D., Vincent, E. B., Liberatore, C. M. and Yutzey, K. E. (1998). A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. *Development* **125**, 4461-4470.
Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S. and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr. Biol.* **11**, 951-961.
Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.
Tada, M., O'Reilly, M. A. and Smith, J. C. (1997). Analysis of competence and of Brachyury autoinduction by use of hormone-inducible Xbra. *Development* **124**, 2225-2234.
Terami, H., Hidaka, K., Katsumata, T., Iio, A. and Morisaki, T. (2004). Wnt11 facilitates embryonic stem cell differentiation to Nkx2.5-positive cardiomyocytes. *Biochem. Biophys. Res. Commun.* **325**, 968-975.
Tzahor, E. (2007). Wnt/beta-catenin signaling and cardiogenesis: timing does matter. *Dev. Cell* **13**, 10-13.
Ueno, S., Weidinger, G., Osugi, T., Kohn, A. D., Golob, J. L., Pabon, L., Reinecke, H., Moon, R. T. and Murry, C. E. (2007). Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **104**, 9685-9690.
Xin, M., Davis, C. A., Molkentin, J. D., Lien, C. L., Duncan, S. A., Richardson, J. A. and Olson, E. N. (2006). A threshold of GATA4 and GATA6 expression is required for cardiovascular development. *Proc. Natl. Acad. Sci. USA* **103**, 11189-11194.
Zhao, R., Watt, A. J., Battle, M. A., Li, J., Bondow, B. J. and Duncan, S. A. (2008). Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. *Dev. Biol.* **317**, 614-619.

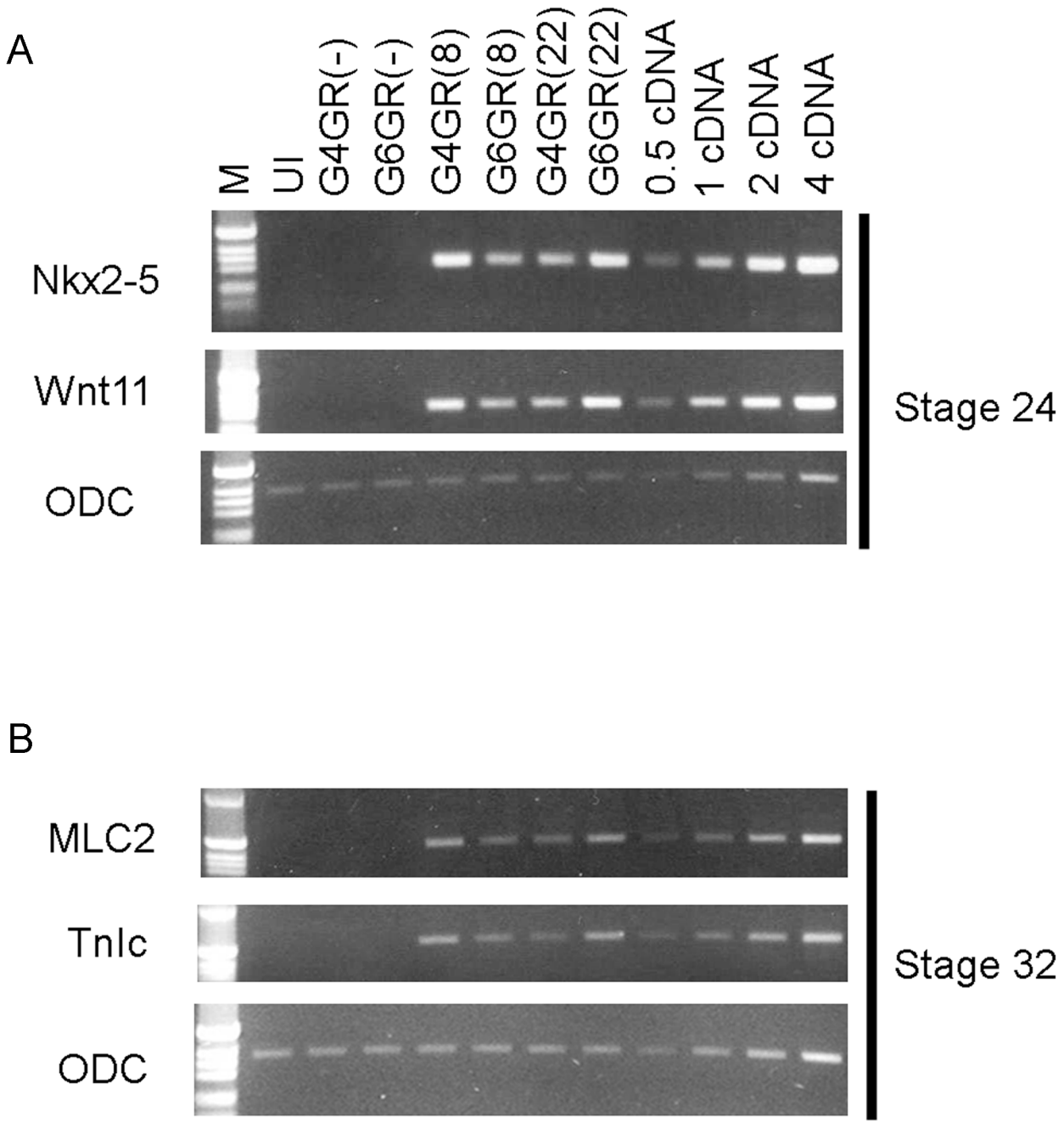
A



B

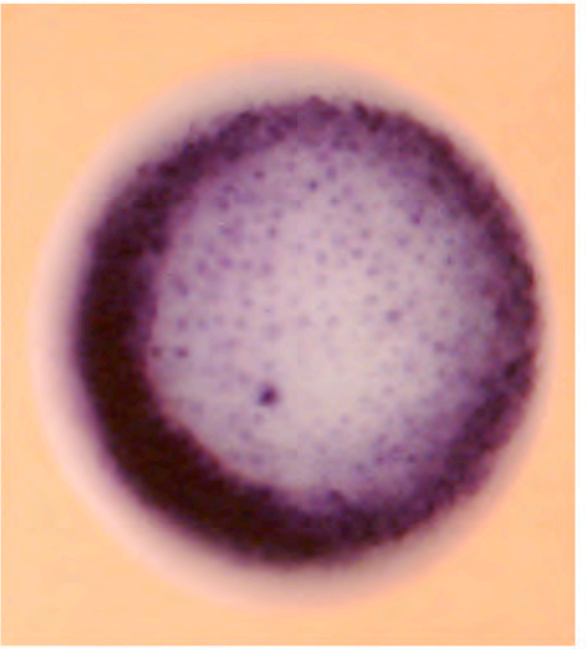


Afouda et al. Fig. S1

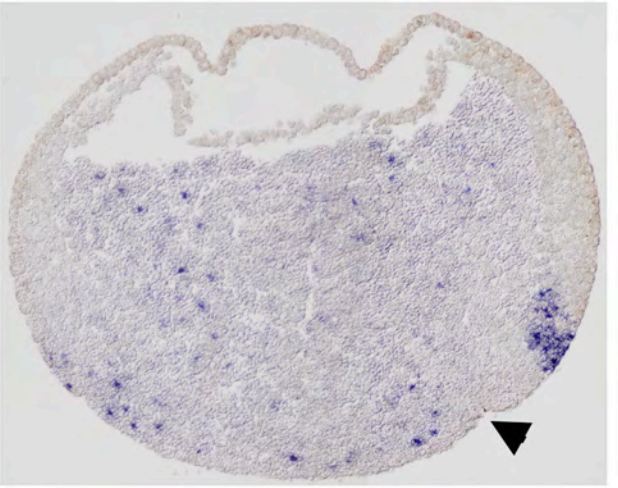


Afouda et al. Fig. S2

A



B



Afouda et al. Fig. S3