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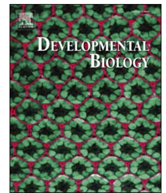

Imaging early embryonic calcium activity with GCaMP6s transgenic zebrafish

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ABSTRACT

Intracellular Ca^{2+} signaling regulates cellular activities during embryogenesis and in adult organisms. We generated stable *Tg[βactin2:GCaMP6s]^{stl351}* and *Tg[ubi:GCaMP6s]^{stl352}* transgenic lines that combine the ubiquitously-expressed Ca^{2+} indicator GCaMP6s with the transparent characteristics of zebrafish embryos to achieve superior *in vivo* Ca^{2+} imaging. Using the *Tg[βactin2:GCaMP6s]^{stl351}* line featuring strong GCaMP6s expression from cleavage through gastrula stages, we detected higher frequency of Ca^{2+} transients in the superficial blastomeres during the blastula stages preceding the midblastula transition. Additionally, GCaMP6s also revealed that dorsal-biased Ca^{2+} signaling that follows the midblastula transition persisted longer during gastrulation, compared with earlier studies. We observed that dorsal-biased Ca^{2+} signaling is diminished in ventralized *ichabod/β-catenin2* mutant embryos and ectopically induced in embryos dorsalized by excess β-catenin. During gastrulation, we directly visualized Ca^{2+} signaling in the dorsal forerunner cells, which form in a Nodal signaling dependent manner and later give rise to the laterality organ. We found that excess Nodal increases the number and the duration of Ca^{2+} transients specifically in the dorsal forerunner cells. The GCaMP6s transgenic lines described here enable unprecedented visualization of dynamic Ca^{2+} events from embryogenesis through adulthood, augmenting the zebrafish toolbox.

1. Introduction

Ca^{2+} ion plays an important role as a second messenger to regulate cellular activity during embryogenesis and in adult organisms. An increase in intracellular Ca^{2+} concentration is generated via a receptor-mediated Ca^{2+} influx from the external space or through Ca^{2+} release from internal stores (Berridge, 1993; Clapham, 1995; Streb et al., 1983). Once intracellular Ca^{2+} concentration is elevated, Ca^{2+}-sensitive proteins, including calmodulin-dependent kinase, protein kinase C, and nuclear factor of activated T cells, can be activated to trigger different cellular responses, such as gene transcription, cell motility, and proliferation (Berridge et al., 2003; Clapham, 2007; De Koninck and Schulman, 1998; Dolmetsch et al., 1998; Gallo et al., 2006; Li et al., 1998; Oancea and Meyer, 1998).

Ca^{2+} signaling is involved in the control of many aspects of early development, including egg activation, cell cleavage, axial patterning, and morphogenesis (Webb and Miller, 2003; Whitaker, 2006). Ca^{2+} waves that propagate over the cell or the embryo were first described during fertilization in Medaka fish and sea urchin (Gilkey et al., 1978; Steinhardt et al., 1977). Subsequent studies suggested Ca^{2+} waves during fertilization are conserved in other organisms (Dumollard and Sardet, 2001; Lee et al., 1999; Runft et al., 2002; Uchida et al., 2000). Following fertilization, several aspects of embryonic cleavages are also regulated by Ca^{2+} signaling, including mitotic chromosome separation, nuclear envelope breakdown, and cytokinesis (Chang and Meng, 1995; Groigno and Whitaker, 1998; Miller et al., 1993; Parry et al., 2005). At later developmental stages, there is evidence that Ca^{2+} signaling is essential in axial patterning and cell migration (Blaser et al., 2006; Kume et al., 1997; Slusarski et al., 1997a; Wallingford et al., 2001; Westfall et al., 2003a). Recent studies also implicate Ca^{2+} signaling in the specification of left-right asymmetry during vertebrate embryogenesis (Garic-Stankovic et al., 2008; McGrath et al., 2003; Sarmah et al., 2005; Schneider et al., 2008; Takao et al., 2013; Yuan et al., 2015).

The translucent nature and rapid external development of the zebrafish embryo make it a particularly attractive model to study Ca^{2+} signaling during vertebrate embryogenesis. One-celled zebrafish zygote undergoes several synchronous cleavages to form a mound of blastomeres atop a large yolk cell (Kimmel et al., 1995). Ca^{2+} signaling

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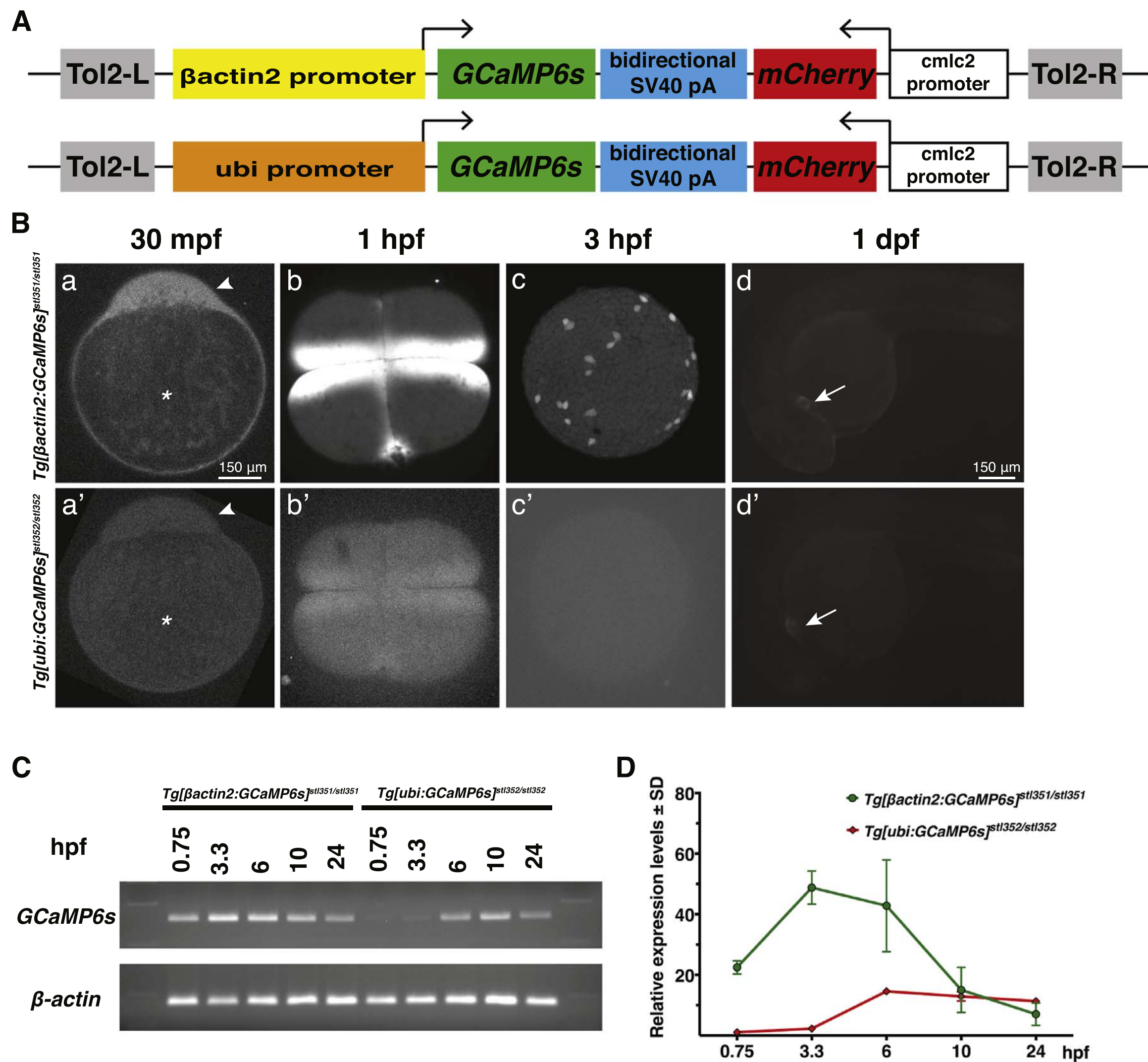

Fig. 1. GCaMP6s expression and fluorescence in *Tg[βactin2:GCaMP6s]*$^{stl351/stl351}$ and *Tg[ubi:GCaMP6s]*$^{stl352/stl352}$ transgenic zebrafish during early embryogenesis. (A) Schematics of the *Tol2[βactin2:GCaMP6s]* or *Tol2[ubi:GCaMP6s]* constructs. (B) GCaMP6s fluorescent confocal microscope images in *Tg[βactin2:GCaMP6s]*$^{stl351/stl351}$ and *Tg[ubi:GCaMP6s]*$^{stl352/stl352}$ embryos at several developmental stages. a, a', d, d', lateral view; b, b', c, c', animal pole view. Asterisks indicate the yolk, and arrowheads point to the blastodisc in a, a'. Arrows point to the heart in d, d'. (C-D) RT-PCR and qRT-PCR analyses of *GCaMP6s* RNA expression levels in *Tg[βactin2:GCaMP6s]*$^{stl351/stl351}$ and *Tg[ubi:GCaMP6s]*$^{stl352/stl352}$ embryos in the course of embryogenesis. The qRT-PCR results were normalized to *β-actin*. Error bars represent standard deviation; N=3.

is essential for cytokinesis at these cleavage stages, as injection of Ca^{2+} chelator, BAPTA, inhibits cytokinesis (Chang and Meng, 1995). Subsequent reports demonstrated that localized Ca^{2+} transients accompany initiation, propagation, and deepening of the cytokinetic furrow during the early cleavages (Webb et al., 1997). At about 64- to 128-cell stage, a different pattern of Ca^{2+} signaling emerges in the superficial blastomeres that form the enveloping layer (EVL). Transient increases of Ca^{2+} activity in the cytoplasm of EVL cells, or Ca^{2+} transients, occur uniformly across the EVL until midblastula transition (MBT) at 3 h post fertilization (hpf), when they display a transient dorsal bias, becoming barely detectable an hour later (Ma et al., 2009; Reinhard et al., 1995). Several studies have shown that disruption of Ca^{2+} release during the early blastula stage preceding MBT leads to dorsalized phenotypes, and revealed an essential role of Ca^{2+} signaling in negatively regulating β-catenin, a key mediator of embryonic axis specification (Westfall et al., 2003b; Wu et al., 2012). Similar perturbations of Ca^{2+} release performed during gastrulation implicated Ca^{2+} signaling in normal behavior of dorsal forerunner cells (DFCs), the precursors of the left-right asymmetry organ, and consequently for left-right laterality establishment (Schneider et al., 2008). Additionally, depletion of Ca^{2+} signaling during *Xenopus* gastrulation inhibits convergence and extension movements (Wallingford et al., 2001).

Despite the established importance of Ca^{2+} signaling in embryogenesis, our understanding of its spatiotemporal dynamics is limited as most of the previous studies employed either synthetic Ca^{2+} dyes or bioluminescent protein Aequorin for transient monitoring of Ca^{2+} signaling (Chang and Meng, 1995; Fluck et al., 1991; Reinhard et al., 1995; Slusarski et al., 1997b; Webb et al., 1997). Genetically encoded Ca^{2+} indicators (GECI) afford more stable and cell-type specific tools for long-term monitoring of Ca^{2+} activity (Miyawaki et al., 1997; Romoser et al., 1997). In particular, transgenic animals expressing GECI possess superior potential for imaging Ca^{2+} activity at later developmental stages or in specific cell types (Dreosti et al., 2009; Tallini et al., 2006). However, such GECIs usually suffer from lower sensitivity and slower turnover than commonly used synthetic Ca^{2+} dyes. The recently engineered GECI, GCaMP6s, shows higher sensitivity compared to commonly used synthetic Ca^{2+} dyes in mammalian and zebrafish neurons (Chen et al., 2013), providing an unprecedented tool with which to study Ca^{2+} dynamics *in vivo*. For example, GCaMP6s expressed selectively in Mauthner neurons in transgenic zebrafish enabled analysis of subcellular Ca^{2+} dynamics during startle behavior, revealing that decreased dendritic excitability underlies startle habituation (Marsden and Granato, 2015).

Here we combine the ultra-sensitivity of GCaMP6s together with

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