Topic Introduction

The Use of Cell-Free *Xenopus* Extracts to Investigate Cytoplasmic Events

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Experiments using cytoplasmic extracts prepared from *Xenopus* eggs have made important contributions to our understanding of the cell cycle, the cytoskeleton, and cytoplasmic membrane systems. Here we introduce the extract system and describe methods for visualizing and manipulating diverse cytoplasmic processes, and for assaying the functions, dynamics, and stability of individual factors. These in vitro approaches uniquely enable investigation of events at specific cell cycle states, including the assembly of actin- and microtubule-based structures, and the formation of the endoplasmic reticulum. Maternal stockpiles in extracts recapitulate diverse processes in the near absence of gene expression, and this biochemical system combined with microscopy empowers a wide range of mechanistic investigations.

BACKGROUND

Research utilizing *Xenopus laevis* oocytes, eggs, and cleavage-stage embryos has provided a number of important insights into the fundamental mechanisms of the meiotic and early mitotic cell cycles. The large size of the eggs, the ease of obtaining them in abundant quantities, their synchronous development, and the ability to microinject and analyze them biochemically led to the discovery of an intrinsic cytoplasmic activity with the same periodicity as the cell cycle (Hara et al. 1980; Gerhart et al. 1984). This activity, termed maturation-promoting factor (MPF), was identified as a highly conserved cell cycle regulatory complex containing cyclin-dependent kinase 1 (cdk1) and cyclin B (Maller et al. 1989). Activation of MPF induces both meiosis and mitosis, whereas its loss induces anaphase and subsequent entry into interphase. The fluctuation of MPF in oogenesis and early *Xenopus* embryos is shown in Figure 1. Hormone treatment of immature oocytes induces MPF activity and drives progression through meiosis I and arrest in metaphase of meiosis II. These eggs maintain high MPF due to cytostatic factor (CSF) activity, eventually identified as Emi2 (Rauh et al. 2005; Schmidt 2006). Upon fertilization, the fusion of the sperm and the egg induces a transient increase in calcium levels that inactivates both CSF and MPF. The first somatic cell cycle lasts 75 min, and subsequent cycles last 25 min. Unfertilized eggs can also be induced to exit CSF metaphase arrest by electrical shock or by treatment with a calcium ionophore.

Characterization of MPF in eggs and embryos was transformative for the cell cycle field. However, microinjection limited the variety of experimental perturbations, and the abundance of refractory yolk

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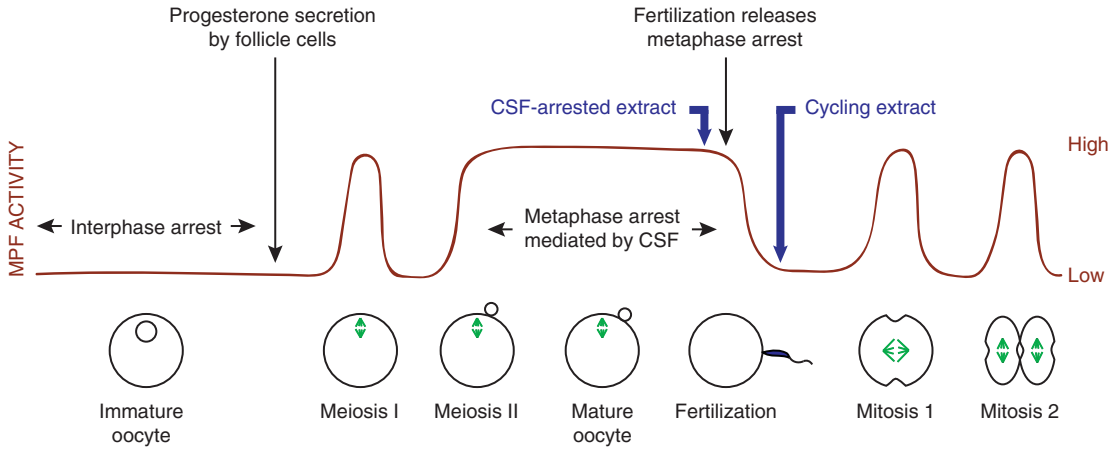

FIGURE 1. Schematic of the cell cycle of the *Xenopus* oocyte and early embryo. Immature oocytes stimulated with progesterone progress through meiosis I and arrest as unfertilized eggs in metaphase of meiosis II with high MPF and CSF activity. Fertilization induces oscillations in MPF activity that peak during mitosis. Points at which extracts are prepared are indicated. (Adapted from Murray [1991].)

platelets made examination of intracellular events in living frog embryos impossible. The development of methods for preparing cell-free extracts from *Xenopus* eggs by Lohka and Masui was a breakthrough that enabled investigation of the cell cycle and downstream events in vitro (Lohka and Masui 1983). By including the calcium chelator EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) in the buffer, egg extracts that stably maintained CSF arrest could be prepared (Lohka and Maller 1985). As a postdoc in Marc Kirschner's lab, Andrew Murray painstakingly optimized extract preparation, and succeeded in preparing synchronized extracts capable of multiple cell cycles in vitro, as well as CSF extracts that could be induced to enter interphase. He used these systems to show that cyclin B synthesis and degradation control MPF activity (Murray and Kirschner 1989; Murray et al. 1989; Murray 1991). Egg extracts were subsequently used to determine that cyclin B is degraded by the ubiquitin pathway (Glotzer et al. 1991), and to study downstream events including DNA replication (Blow and Laskey 1986), microtubule dynamics and spindle assembly (Belmont et al. 1990; Verde et al. 1990; Sawin and Mitchison 1991), and vesicle fusion (Tuomikoski et al. 1989). The protocols introduced here present the latest approaches that illustrate how the extract system can be used to examine a variety of cytoplasmic events (Fig. 2). Extract-based investigation of nuclear events is described elsewhere in this collection.

PREPARING EXTRACTS AND PERFORMING CELL CYCLE AND BIOCHEMICAL ASSAYS

The key to preparing robust egg extracts is to prevent dilution of the cytoplasm by packing dejellied eggs using a low-speed centrifugation step, which is followed by a high-speed spin to crush the eggs and fractionate them so that the cytoplasm can be collected. Protocol: **Preparation of Cellular Extracts from *Xenopus* Eggs and Embryos** (Good and Heald 2018) describes the basic method to prepare CSF-arrested *X. laevis* extracts and is adapted for eggs from the smaller *Xenopus* species, *X. tropicalis*, as well as for preparation of metaphase-arrested embryo extracts. These approaches have facilitated the identification of factors that modulate spindle and nuclear size across species and during the reductive divisions of embryonic development (Levy and Heald 2010; Loughlin et al. 2011; Wilbur and Heald 2013). In Protocol: **Robustly Cycling *Xenopus laevis* Cell-Free Extracts in Teflon Chambers** (Chang and Ferrell 2018), spontaneously cycling extracts are prepared from eggs treated with a calcium ionophore to degrade CSF and induce progression through interphase and into the next cell cycle. Interestingly, the materials to which extracts are exposed strongly affects their activity. The robust Teflon tube system could be used to elucidate how MPF activation propagates through the egg extract

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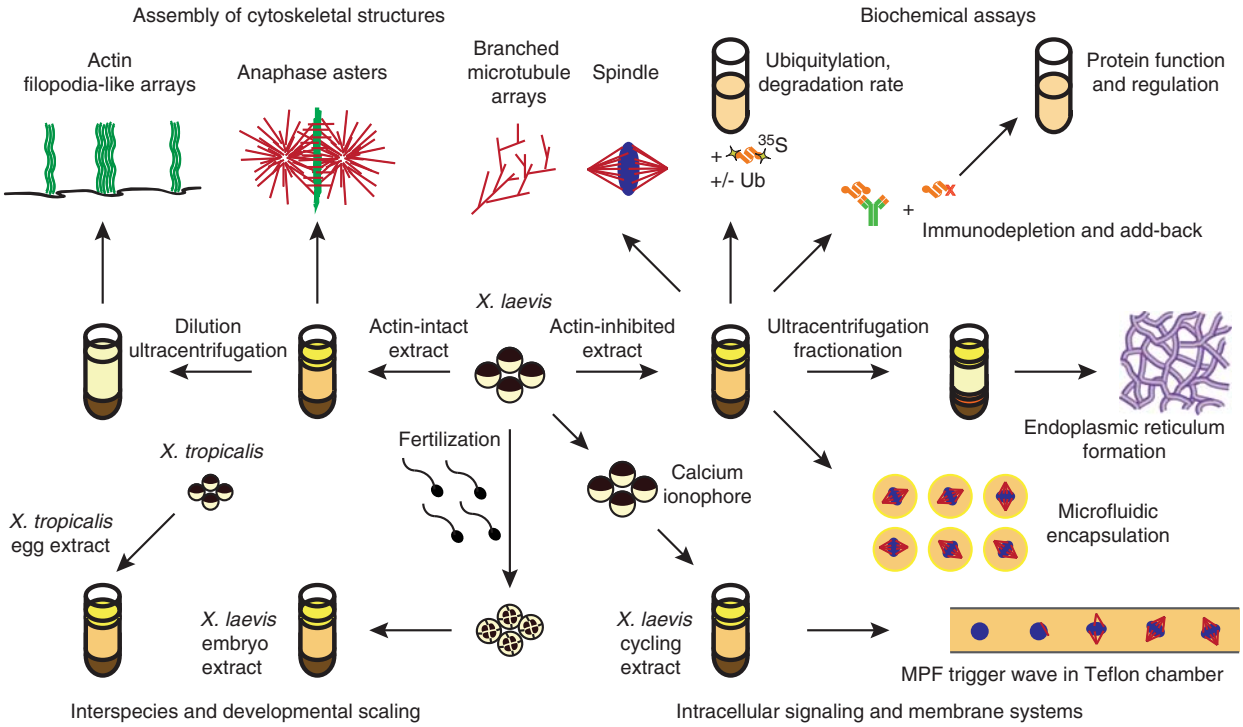

FIGURE 2. Schematic of the cytoplasmic processes reconstituted using the *Xenopus* in vitro systems described in the associated protocols.

in the form of a trigger wave (Chang and Ferrell 2013). Reconfinement of egg extract has emerged as a useful approach to evaluate the effects of compartment volume, shape, and boundary conditions on cellular processes under highly controlled conditions. In Protocol: **Microfluidic Encapsulation of Demembranated Sperm Nuclei in *Xenopus* Egg Extracts** (Oakey and Gatlin 2018), the authors describe how confinement technologies can be applied to generate synthetic cell-like systems, which have uniquely enabled evaluation of the impact of cytoplasmic volume on spindle size (Good et al. 2013; Hazel et al. 2013).

Importantly, the functional roles of the proteins involved in any process reconstituted in egg extracts can be determined by depleting a protein of interest using antibodies coupled to beads. Wild-type and mutant versions can be added back to verify function and test domain activities. Protocol: **Protein Immunodepletion and Complementation in *Xenopus laevis* Egg Extracts** (Jenness et al. 2018) describes how this complementation can be achieved by adding either recombinant proteins or mRNAs that are translated in the extract. This approach has been instrumental in defining the functions of cell cycle and other proteins and their regulatory mechanisms. For example, the amino-terminal 90 amino acids of cyclin B were shown to be required for its ubiquitylation and degradation, and sufficient to confer M-phase-specific degradation when fused to another protein (Glotzer et al. 1991). In Protocol: **Assessing Ubiquitylation of Individual Proteins Using *Xenopus* Extract Systems** (McDowell and Philpott 2018a) and Protocol: **Calculating the Degradation Rate of Individual Proteins Using *Xenopus* Extract Systems** (McDowell and Philpott 2018b), methods are presented that capitalize on the robust ability of the egg extract to induce protein degradation in order to quantitatively measure the ubiquitylation and half-life of exogenously added proteins. In addition to ubiquitylation, other posttranslational modifications, such as phosphorylation, have been characterized using *Xenopus* extract systems. Recent proteomic and phosphoproteomic analyses of *Xenopus* eggs and embryos provide an important resource for cell biologists using extract systems (Wühr et al. 2014, 2015; Peuchen et al. 2017; Presler et al. 2017).

ASSEMBLING CELLULAR CYTOSKELETAL AND MEMBRANE STRUCTURES IN VITRO

The ability to control a switch-like transition between metaphase and interphase, and arrest in either state, has facilitated experiments assessing the effects of cell cycle state on the morphology and dynamics of subcellular structures. Protocol: **Assembly of Spindles and Asters in *Xenopus* Egg Extracts** (Field and Mitchison 2018) provides methods for examining the microtubule cytoskeleton in extracts and illustrates its similarity to structures formed in vivo, as well as the use of fluorescent cytoskeletal proteins. Spindle assembly and the role of motor proteins and other factors have been investigated extensively in egg extracts (e.g., Walczak et al. 1998), and many experiments now focus on elucidating the mechanisms underlying the subprocesses of microtubule organization and dynamics. Protocol: **Dissecting Protein Complexes in Branching Microtubule Nucleation Using Meiotic *Xenopus* Egg Extracts** (Song and Petry 2018) provides methods to assay a key step in spindle assembly using immunodepletion and total internal reflection fluorescence (TIRF) microscopy. This protocol describes how to examine the role of a particular protein complex and investigate its assembly by mass spectrometry analysis of immunoprecipitates. The actin cytoskeleton can also be examined in egg extracts. Prior to 2011, most egg extract preparations utilized cytochalasin to inhibit actin polymerization and prevent cytoplasmic gelation and contraction that complicated analysis by microscopy and biochemistry. Field et al. (2011) showed that bulk contraction driven by actomyosin was cell cycle regulated and specific to metaphase extract. Cytochalasin-free actin-intact egg extract has subsequently been used to investigate the physics of symmetry breaking by actin (Abu Shah and Keren 2014). Strikingly, when combined with lipid bilayers, actin-intact extract could reconstitute cytokinesis signaling from microtubules to the plasma membrane (Nguyen et al. 2014). Methods for preparation of actin-intact egg extracts are described elsewhere (Field et al. 2014, 2017). Protocol: **Filopodia-Like Structure Formation from *Xenopus* Egg Extracts** (Fox and Gallop 2018) describes the assembly of one type of actin architecture, and recapitulates an actin-membrane interaction in vitro. Finally, Protocol: **Endoplasmic Reticulum Network Formation with *Xenopus* Egg Extracts** (Wang et al. 2018) illustrates how endoplasmic reticulum (ER) morphology is affected by cell cycle state, and describes the fractionation and labeling of membranes that can be combined with cytosol to reconstitute the organelle. Egg extract-based assays were instrumental in identifying cellular components that that define ER morphology (Dreier and Rapoport 2000; Voeltz et al. 2006; Hu et al. 2009; Wang et al. 2013).

Over the past several decades, *Xenopus* extracts have contributed to our knowledge in many areas of cell biology, and no other cytoplasmic in vitro system has simultaneously enabled both physiological reconstitution and biochemical approaches. To summarize the key features: (1) Extracts faithfully recapitulate complex cellular events in vitro; (2) The synchronized cell cycle state is preserved and can be controlled; (3) Extracts can easily be manipulated biochemically and observed by fluorescence microscopy; (4) Sufficient volumes can be obtained to perform a variety of assays.

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