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Studying the DNA damage response using *in vitro* model systems

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ABSTRACT

Exogenous and endogenous insults continuously damage DNA. DNA damage must be detected in order to prevent loss of vital genetic information. Cells respond to DNA damage by activating checkpoint pathways that delay the progression through the cell cycle, promote DNA repair or induce cell death. A regulatory network of proteins has been identified that participate in DNA damage checkpoint pathways. Central to this network are ATM, ATR and the Mre11/Rad50/Nbs1 (MRN) complex. Detailed biochemical analysis of ATM, ATR and the MRN dependent DNA damage responses has taken advantage of several *in vitro* model systems to understand the detailed mechanisms underlying their function. Here we describe some recent findings obtained analysing these pathways using *in vitro* model systems. In particular we focus on the studies performed in the *Xenopus laevis* egg cell free extract, which recapitulates the DNA damage response in the context of the cell cycle.

1. Introduction

In order to preserve genomic stability, eukaryotic cells harbour highly flexible and integrated signalling systems that facilitate the sensing of DNA damage and subsequent responses to promote repair or cell death. Concomitantly, such signalling may modulate cell cycle progression to support the process of repair. The process of cell cycle arrest as a consequence of DNA damage signalling is known as the 'checkpoint response'. Checkpoint responses are intrinsically linked to the cell cycle and as such some of the first experiments that supported the concept of checkpoints in response to DNA damage were performed in cycling eukaryotic cell systems such as yeasts and cells derived from mammalian hosts. The genetic malleability of yeast systems and the ease with which cell cycle progression can be observed makes yeast a powerful tool in the study of the cell cycle. Additionally, the virtue of the genetic similarity of various mammalian cell systems combined with advances in technology of cell biology applications has paved the way for furthering our knowledge of the eukaryotic DNA damage response. However, despite the unequivocal value of such systems, the study of this response has gained important insights regarding the molecular mechanisms underlying their function via the exploitation and development of several important *in vitro* systems. *In vitro* systems make use of molecular and biochemical techniques to recapitulate protein function, protein–protein and protein–DNA interactions that provide a profound insight into various aspects of DNA damage signalling.

2. *In vitro* systems to examine DNA damage responses

2.1. Purified protein systems

Biochemical techniques are at the core of understanding many aspects of protein structure, activity and function and indeed the cellular response to DNA damage has been subjected to intense *in vitro* biochemical characterisation. The ultimate goal of reconstituted biochemical systems is to reproduce, at least in part, complex biochemical reactions with purified components in an attempt to study the role of the single players in the overall pathway. Purified proteins are of pivotal importance, providing a basis for understanding precise molecular interactions and frequently provide unequivocal support toward models proposed following observations in other systems. The knowledge of several components of checkpoint pathways has allowed in the recent years the reconstitution of many important reactions with purified recombinant proteins especially concerning the early steps of DNA damage recognition and initial checkpoint activation. The definition of such systems has led to the discovery of detailed molecular mechanisms behind fundamental checkpoint pathways [1–7]. However, it is clear that although powerful, this approach cannot account for yet undefined and unknown steps in the complex processes that it tries to reproduce, especially when some of the players are yet to be discovered. In addition, large proteins are often difficult to obtain in useful amounts (Table 1). An important complement to purified systems is the use of cell extracts capable of recapitulating

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complex biological events in the test tube in a more physiological context.

2.2. Yeast, Drosophila and mammalian cell extracts

Cell extracts from yeast, Drosophila and mammalian sources (such as human and mouse) can be used to partially reconstitute DNA replication, DNA repair and checkpoint responses. There are several advantages to this, in that extracts from mutant isogenic lines can be compared and the ease of genetic manipulation available to yeast experimentation can be exploited in an *in vitro* system. Mammalian cell extracts have been used quite extensively to study aspects of DNA repair. Such assays frequently combine refined protein purification techniques to examine the specific activities of purified proteins in a system that reconstitutes repair processes on synthetic DNA molecules containing specific lesions [8–12]. However, it has been more difficult to define systems that recapitulate DNA damage checkpoints. This is likely due to technical issues such as the ability to obtain functional protein mixtures and the relatively limited protein yield that can be obtained from such extract systems (Table 1). A further problem with such purified systems is the limited ability to recreate complex phenomenon such as the cell cycle. As such, these systems have not been utilized as successfully as the *Xenopus laevis* egg extract to study checkpoint responses in which cell cycle context is an important and intrinsic factor.

2.3. Xenopus laevis egg extract

The *Xenopus laevis* egg extract provides a platform that recapitulates chromatin formation, nuclear assembly, DNA replication, mitotic spindle assembly and chromosome segregation. *Xenopus* eggs contain an abundance of proteins required to drive cell cycle progression and multiple rounds of cell division upon fertilisation in the absence of transcription. Consequently, biological reactions are driven by protein–protein interactions, protein post-translational modifications and enzymatic activities. Historically, many of the initial observations regarding aspects of early embryonic cell cycle events were made from the study of *Xenopus* egg cell division, such as the oscillating levels of maturation promoting factor (MPF) in meiotic and mitotic cell cycles [13,14] or from similar systems such as fertilized sea urchin eggs [15]. The desire for a more experimentally flexible system to study biochemical aspects of the cell cycle led to the development of the sensitive egg extract cell free system [16–24]. While over the years many protocols have refined the way in which extract is prepared and detailed the production of various types of extract the fundamental process is the same for most derivations. Essentially, eggs are crushed by centrifugation creating a density separation that allows the extraction of egg cytoplasm. The egg cytoplasm is uniquely able to undergo rapid and spontaneous oscillation of cyclin dependent kinase (Cdk) activity driving the extract into consecutive rounds of mitosis and S-phase [25]. Demembranated sperm nuclei derived from the male testis, when added to the egg extract, undergo complete rounds of chromatin and nuclear membrane assembly, semi-conservative replication, nuclear envelope breakdown and mitotic chromosome condensation [25]. Multiple passages through the cell cycle are achievable using this system and as such, egg extracts have proven a powerful tool for the *in vitro* study of both DNA replication and cell cycle progression [17,18,26]. A more elaborate version of the *Xenopus* egg extract entails the processing of nuclei assembled in egg extract to produce a highly enriched fraction of nuclear proteins (nucleoplasmic extract or NPE) that allows the replication of double-stranded DNA (dsDNA) templates in the absence of a nuclear membrane [27]. The egg extract mostly recapitulates rapid embryonic cell cycle events taking place in the absence of transcription and as such it constitutes an approximation of somatic cell cycle processes. However, embryonic cell cycle events can be considered closer to the ones taking place in fast proliferating and poorly differentiated cancer cells.

The intrinsic features of egg extract provide some clear advantages and a few disadvantages for the study of important biological processes. A major advantage is the high protein concentration and the selective enrichment of cell cycle factors, which ensures that proteins that might be present in limited amount in other systems are instead well represented in the egg extract. In addition, the high degree of genetic conservation between *Xenopus* and mammalian

Table 1
The pros and cons of *in vitro* systems.

Advantages	Disadvantages
Xenopus egg cell free extract	
Eggs can be obtained in large quantities to make egg extract	*Xenopus* specific depleting antibodies need to be generated in high amount
Egg extract provides a rich source of biologically active proteins and membrane components	Wild type or mutated soluble protein components must be purified in high amount to reconstitute depleted extract
Egg extract is capable of recapitulating the cell cycle events such as genomic DNA replication and mitosis	'Quality' of extract can vary and initial egg quality is essential
Proteins can be depleted or added in allowing a biochemical 'knock-out' or 'knock-in' approach	*Xenopus laevis* genome has not been completely sequenced (However the fully sequenced *X. tropicalis* genome is available)
Egg extract retains the ability for post-translational modification of proteins	
High degree of conservation of pathways from *Xenopus* to mammals	
Yeast, mammalian, Drosophilia cell free extract	
Genomic modification of yeast strains is relatively quick and easy	Many mammalian pathways are not represented in yeast i.e. apoptotic pathways
Drosophila are amenable to mutational analysis	Cell cycle progression in vitro is difficult to achieve
Genetic similarity of mammalian extracts and cell cultures from patients available	
Extract systems represent powerful systems for examining DNA repair processes	
Purified protein systems	
Structural studies can yield information about interacting partners, domains and candidate regions for post-translational modification	Large proteins are relatively difficult to purify
The function of single proteins with defined stoichiometries can be analysed	Biochemical analysis often requires large quantities of protein, which can be difficult to achieve
	Unknown steps in the pathway cannot be reconstituted