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REVIEW

CRISPR-Cas tools to study gene function in cytokinesis

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ABSTRACT

Cytokinesis is the process that separates a cell into two daughter cells at the end of mitosis. Most of our knowledge of cytokinesis comes from overexpression studies, which affects our interpretation of protein function. Gene editing can circumvent this issue by introducing functional mutations or fluorescent probes directly into a gene locus. However, despite its potential, gene editing is just starting to be used in the field of cytokinesis. Here, we discuss the benefits of using gene editing tools for the study of cytokinesis and highlight recent studies that successfully used CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) technology to answer critical questions regarding the function of cytokinesis proteins. We also present methodologies for editing essential genes and discuss how CRISPR interference (CRISPRi) and activation (CRISPRa) can enable precise control of gene expression to answer important questions in the field. Finally, we address the need for gene editing to study cytokinesis in more physiologically relevant contexts. Therefore, this Review provides a roadmap for gene editing to be used in the study of cytokinesis and other cellular processes.

KEY WORDS: CRISPR, RhoA, Actomyosin, Cytokinesis, Gene editing

Introduction

Cytokinesis occurs at the end of mitosis to physically separate a cell into two daughter cells. A RhoA-dependent actomyosin ring ingresses to pinch in the membrane between the segregating chromosomes (Fig. 1A). The contractile ring is spatiotemporally controlled by multiple spindle-dependent and -independent pathways to ensure that cells inherit the correct fate determinants and genetic content (Fig. 1B). Failure to do so can cause changes in ploidy or cell fate, which can lead to the development of diseases, including cancer (Fededa and Gerlich, 2012; Green et al., 2012; Lacroix and Maddox, 2012; D'Avino et al., 2015; Pollard and O'Shaughnessy, 2019). Active, GTP-bound RhoA (RhoA-GTP), promotes the assembly and ingression of the contractile ring by regulating effectors, including formins and Rho-associated protein kinases (ROCK1 and ROCK2), to form actin filaments and activate myosin, respectively (Fig. 1C; Piekny et al., 2005; Pollard and O'Shaughnessy, 2019). Epithelial cell transforming 2 (Ect2) is the main guanine nucleotide exchange factor (GEF) that generates RhoA-GTP at the equatorial cortex and requires binding to Cyk4 (also known as MgcRacGAP and RACGAP1) to be active (Yüce et al., 2005; Wolfe et al., 2009; Gómez-Cavazos et al., 2020). Active RhoA recruits anillin, which is a scaffold protein that tethers the contractile ring to the plasma membrane (Piekny et al., 2005; Piekny and Glotzer, 2008; Green et al., 2012; Sun et al., 2015). Recent studies have shown that anillin positively feeds back to reinforce the division plane through the recruitment of phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2) and retention of active RhoA, which preferentially binds to PIP_2 (Budnar et al., 2019). After ingression, the contractile ring and central spindle transition into a midbody, which is the site of abscission (Fig. 1A; Fededa and Gerlich, 2012; Green et al., 2012; Kechad et al., 2012; El Amine et al., 2013; Mierzwa and Gerlich, 2014). The different stages of cytokinesis have been described extensively in multiple reviews (see, among others, Piekny et al., 2005; Fededa and Gerlich, 2012; Green et al., 2012; Mierzwa and Gerlich, 2014; D'Avino et al., 2015; Glotzer, 2017; Basant and Glotzer, 2018; Pollard and O'Shaughnessy, 2019).

New methods are needed to help further advance the field of cytokinesis. Most of our knowledge has been generated using RNA interference (RNAi) to generate loss-of-function phenotypes and exogenous constructs to study protein localization and function. In addition, due to their amenability for *in vitro* use, many studies were done using HeLa (human) or S2 (*Drosophila melanogaster*) cells, which are cancerous and/or lack characteristics of cells *in vivo*, whereas others used the one-cell *Caenorhabditis elegans* embryo, *Xenopus laevis* or echinoderm embryos, the findings from which may reflect mechanisms unique to undifferentiated cells. Gene editing tools enable studies using a broad range of systems. By introducing functional mutations or visualization probes directly into a gene locus, cytokinesis regulators can be studied in a plethora of physiological contexts to reveal important differences between cell types. Advancements in gene editing, and particularly CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) tools, should facilitate the generation of new knowledge in the field of cytokinesis.

Here, we review recent advances in cytokinesis, with an emphasis on key questions related to ring assembly, positioning and ring-to-midbody transition. We also discuss the benefits of using gene editing tools to generate loss-of-function mutations, separation-of-function mutations and endogenous tags for protein visualization. In particular, we highlight studies that have successfully used CRISPR-Cas technology to solve critical questions regarding the function of cytokinesis proteins. We also present methodologies for the editing of essential genes, and how CRISPR interference (CRISPRi) and activation (CRISPRa) can enable precise control of gene expression. Finally, we address the need for gene editing to study cytokinesis in a broader range of physiological contexts.

Cytokinesis – where are we now and where are we headed?

Cytokinesis encompasses multiple steps, which initiate in anaphase. The central dogma in cytokinesis is that the anaphase spindle provides cues to direct contractile ring assembly in the equatorial plane. Moving the spindle to a new location generates active RhoA and causes a new furrow to form in the location that bisects it (Rappaport, 1985; Bement et al., 2005). Centralspindlin [a complex between Cyk4 and MKLP1 (also known as KIF23)] regulates central spindle assembly and recruits Ect2 (Yüce et al., 2005). In human

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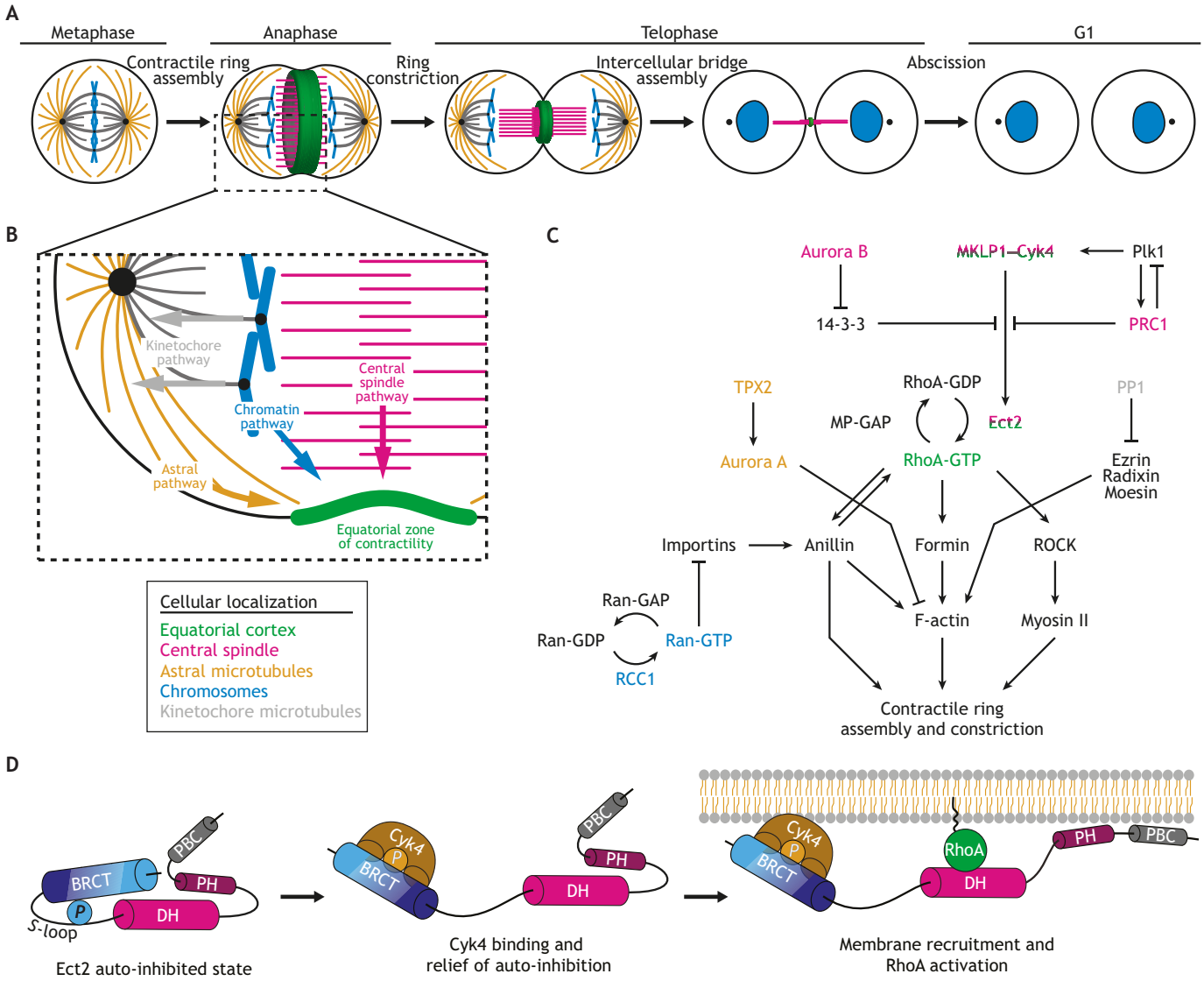

Fig. 1. Overview of cytokinesis. (A) A schematic showing a cell undergoing cytokinesis. During anaphase, cortical regulators accumulate at the equatorial cortex and assemble into a contractile ring. As cells progress through telophase, the ring constricts to pinch in the overlying plasma membrane, followed by formation of an intercellular bridge and midbody to promote abscission. (B) A region of the cell is shown with a formed contractile ring to highlight the multiple pathways regulating contractile ring assembly. These include signals emanating from the central spindle (pink), astral microtubules (yellow), chromatin (blue) and kinetochores (gray). (C) The molecular components of the pathways regulating ring assembly are shown. These pathways regulate the generation and localization of active RhoA or regulate downstream components at the equatorial cortex (proteins in green text). Active RhoA recruits and activates effectors (anillin, formin and ROCK proteins) to generate actomyosin filaments and assemble the ring. A central spindle pathway (proteins in pink text) promotes the recruitment and activation of Ect2 (a RhoA GEF) to the central spindle and equatorial cortex through Cyk4 binding. An astral microtubule pathway (proteins in yellow text) also exists, wherein microtubules recruit TPX2, which activates Aurora A kinase to clear F-actin at the polar cortex. A chromatin pathway (proteins in blue text) relies on a gradient of Ran-GTP that forms around chromatin, and generates an inverse gradient of importins that control the localization and function of anillin at the equatorial cortex. A kinetochore-directed pathway (PP1; gray) induces relaxation of the polar cortex also by clearing F-actin, via PP1-mediated dephosphorylation of ERM proteins. Proteins in black font are broadly regulated or localized, are regulated by multiple pathways or are regulated in ways that are beyond the scope of this figure. (D) A schematic showing the current model for Ect2 activation. The Cdk1-phosphorylated S loop of Ect2 interacts with the BRCT domains (blue) and partially blocks the DH domain (pink), whereas the PH domain (maroon) also inhibits the DH domain. Dephosphorylation allows the BRCT domains to interact with Plk1-phosphorylated Cyk4 (brown), whereas phospholipid binding to the PH domain likely relieves autoinhibition of the DH region to generate active RhoA (green).

cells, Ect2 requires binding to Cyk4 to activate RhoA, and the contractile ring fails to form in cells depleted of Ect2 or Cyk4 (Yüce et al., 2005; Zhao and Fang, 2005; Kamijo et al., 2006; Nishimura and Yonemura, 2006). In metaphase, Cdk1 (cyclin-dependent kinase 1) phosphorylates site(s) in the S loop of Ect2, which inhibits its function (Fig. 1D). Autoinhibition occurs because this phosphorylated site binds to the N-terminal BRCT (BRCA1 C-terminus) domains, causing conformational changes that hinder the C-terminal Dbl homology (DH) domain required for GEF activity (Fig. 1D; Hara et al., 2006; Niiya et al., 2006). This mechanism restricts Ect2 activity to the time when it is required. When cells enter anaphase, Ect2 is dephosphorylated and Cyk4 binding helps relieve autoinhibition. Cyk4 also requires phosphorylation by Polo-like kinase 1 (Plk1) to bind Ect2 (Fig. 1D; Yüce et al., 2005; Hara et al.,

2006; Niiya et al., 2006; Petronczki et al., 2007; Burkard et al., 2009; Wolfe et al., 2009). As the central spindle extends towards the cortex, Ect2 is enriched at the overlying membrane (Su et al., 2011; Lekomtsev et al., 2012; Kotynkova et al., 2016). Both Cyk4 and Ect2 have phospholipid-binding domains required for their function, and studies suggest that the role of the central spindle may be to position the Cyk4–Ect2 complex rather than to control its activity (Frenette et al., 2012; Lekomtsev et al., 2012; Basant et al., 2015). In multiple cell types, active RhoA (RHO-1 in *C. elegans*) localizes to a broader zone after the depletion of central spindle proteins, such as protein regulator of cytokinesis 1 [PRC1; spindle-defective protein 1 (SPD-1) in *C. elegans*] or MKLP1 (ZEN-4 in *C. elegans*) (Mollinari et al., 2005; Yüce et al., 2005; Adriaans et al., 2019). Recent studies have proposed that the central spindle positions Cyk4–MKLP1 complexes to activate Ect2 in proximity to the equatorial cortex (Mishima et al., 2002, 2004; Adriaans et al., 2019). Plk1 is activated by PRC1 at the central spindle, where it could access Cyk4 for phosphorylation. Phosphorylated Cyk4 could then form a complex with Ect2 that is 'released' to the overlying cortex to generate active RhoA.

Recent studies have provided new insight as to how the Cyk4–Ect2 complex is regulated. Structural data have revealed that the pleckstrin homology (PH) domain of Ect2 could autoinhibit the DH domain, and binding to active RhoA and/or phospholipids relieves this inhibition (Chen et al., 2020). In *C. elegans*, one of the BRCT domains of ECT-2 has a conserved basic surface that binds to phospho-CYK-4, which recruits CYK-4 to the membrane to form a stable complex required to generate active RHO-1 (Fig. 1D; Gómez-Cavazos et al., 2020). However, Ect2 localizes to the central spindle in human cells (Yüce et al., 2005; Cabernard et al., 2010; Su et al., 2011; Basant et al., 2015; Basant and Glotzer, 2018). Thus, we propose a model where human Ect2 binds to phospho-Cyk4, which helps to partially relieve autoinhibition and whose membrane recruitment helps fully activate Ect2 (Fig. 1D). Another pathway involving Aurora B kinase frees Cyk4–MKLP1 complexes from 14-3-3ζ (partitioning defective 5, PAR-5 in worms) at the membrane and may function redundantly with the spindle-directed Plk1 pathway described in cultured human cells (Basant et al., 2015; Adriaans et al., 2019). However, the requirement for these mechanisms likely varies depending on the cell type and organism. Importantly, these models do not provide an explanation for how active RhoA is spatially restricted to the equatorial cortex.

Several studies have proposed that the astral microtubules that extend from centrosomes to the cortex could prevent cortical regulators from accumulating at the poles (Zanin et al., 2013; van Oostende Triplet et al., 2014). Astral microtubules may be acting to remove these cortical regulators in the absence of active RhoA outside the equatorial plane (Tse et al., 2011; van Oostende Triplet et al., 2014). Astral targeting protein for Xklp2 (TPX2, TPXL-1 in worms) contributes to the polar clearance of anillin (ANI-1 in worms) and F-actin by activating Aurora A kinase, although the mechanism is not clear (Fig. 1C; Mangal et al., 2018). Astral microtubules also act in concert with M-phase GTPase-activating protein [MP-GAP, also known as ARHGAP11A; Rho GTPase activating protein 3 and 4 (RGA-3 and 4) in worms], which is a globally localized negative regulator of RhoA (Zanin et al., 2013). Overall, central spindle and astral microtubules take on different roles by virtue of their position – being bundled by centralspindlin and PRC1, and controlling where Ect2 is active or removing components from the cortex (Inoue et al., 2004; Nishimura and Yonemura, 2006; Foe and von Dassow, 2008), respectively.

Spindle-independent pathways also regulate the cortex via chromatin, centrosomes and kinetochores (Canman et al., 2000, 2003; von Dassow et al., 2009; Cabernard et al., 2010; Kiyomitsu and Cheeseman, 2013; Zanin et al., 2013; Rodrigues et al., 2015; Beaudet et al., 2017, 2020; Ozugergin and Piekny, 2021). A kinetochore-derived pathway induces relaxation of the polar cortices in cultured mammalian and *Drosophila* cells (Rodrigues et al., 2015). This is accomplished by a kinetochore-tethered protein phosphatase 1 (PP1)–protein phosphatase 1 regulatory subunit 7 (PPP1R7; Sds22 in flies) complex that dephosphorylates the ezrin, radixin and moesin (ERM) proteins, which anchor actin to the plasma membrane. This reduces membrane-associated F-actin and induces relaxation of the polar cortex (Rodrigues et al., 2015). Several studies also revealed a role for chromatin-associated active Ran (Ras-related nuclear protein) in coordinating the position of cortical regulators with segregating chromosomes (Kiyomitsu and Cheeseman, 2013; Rodrigues et al., 2015; Beaudet et al., 2017). Active GTP-bound Ran (Ran-GTP) is high around chromatin as its GEF, regulator of chromosome condensation 1 (RCC1), is bound to histones, whereas Ran GTPase-activating protein 1 (RanGAP1) is enriched in the cytosol. Active Ran displaces importins from nuclear localization signal (NLS)-containing proteins, which forms an inverse gradient where importins are free to bind to NLS-containing proteins at the cortex (Ozugergin and Piekny, 2021). We found that the NLS of anillin is required for importin-β binding, and for its cortical localization and function (Fig. 1C; Beaudet et al., 2017, 2020). Overall, these different pathways controlling the function of cortical regulators ensure robust cytokinesis, and some pathways could be favored over others depending on cell polarity, fate or ploidy.

Other aspects of cytokinesis, including ring constriction and ring-to-midbody transition, are not fully understood (Pollard, 2017; Fremont and Echard, 2018; Liu and Robinson, 2018; Nguyen and Robinson, 2020). Specifically, it is not clear how forces are generated for ring constriction, and how they overcome stiffness and tension (O'Shaughnessy and Thiyagarajan, 2018; Osório et al., 2019; Pollard and O'Shaughnessy, 2019). Early models postulated that the ring is formed by contractile units, and the number of units correlates with ring size and ingression speed (Carvalho et al., 2009). However, different cells will have different cortical properties, forces and levels of cortical regulators, all of which could influence ingression speed. A recent model proposed that constriction is resisted by the compression of membrane microdomains, and that these microdomains are progressively cleared by anillin–septin complexes (Carim et al., 2020). This model suggests that the loss of plasma membrane contributes to ingression, contradicting the convention that membrane expansion is needed for ingression (Pollard, 2017; Fremont and Echard, 2018). Mathematical modeling is helping to address some of these issues, combined with biophysical studies of force generation and regulation (Dorn et al., 2016; Cortes et al., 2018; Descovich et al., 2018; Leite et al., 2019). In addition, it is not clear what drives the turnover of actomyosin filaments required for the ring-to-midbody transition. F-actin disassembly presumably leads to a transition in complexes that tether actomyosin to those regulating midbody formation. In *Drosophila* S2 cells and the one-cell *C. elegans* embryo, ring components including lipids, RhoA, anillin and septin, are shed as the ring transitions to the midbody (El Amine et al., 2013; Green et al., 2013; El-Amine et al., 2019; Carim et al., 2020). More recently, it was shown that F-actin depolymerization is induced by the protein microtubule-associated monooxygenase, calponin and LIM domain containing 1 (MICAL1) in the intercellular bridge (Fremont et al., 2017). Overall, many questions remain in our understanding of cytokinetic processes, and their answers may differ depending on the organism and cellular context.

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Using CRISPR-Cas knockouts to study cytokinesis genes

CRISPR-Cas gene editing tools have revolutionized our ability to manipulate genes to study their function. CRISPR-Cas9 consists of an RNA-guided endonuclease (Cas9) in complex with a single guide RNA (sgRNA) that directs Cas9 cleavage (Fig. 2A; Cong et al., 2013; Mali et al., 2013; Wang et al., 2016; Pickar-Oliver and Gersbach, 2019). Cas9 double-stranded breaks (DSBs) are generally repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR; Scully et al., 2019). NHEJ errors occasionally introduce indel mutations, which can result in frame-shifting loss-of-function mutations (Fig. 2A; Cong et al., 2013). Knockouts can also be generated by creating two DSBs around a gene which are then joined by NHEJ without the intervening sequence (Chen et al., 2014). The HDR pathway repairs Cas9-induced DSBs using a homologous DNA template (Wright et al., 2018), which can be provided to introduce the desired mutations or insertions (Verma et al., 2017). NHEJ is the predominant repair pathway in most animal cells, and it is preferred to generate loss-of-function alleles.

The generation of loss-of-function alleles using CRISPR-Cas9 has been used to study the function of some cytokinesis proteins (Liu et al., 2016; Xu et al., 2019; Yamamoto et al., 2019; Peterman et al., 2020; Uretmen Kagiali et al., 2019) but has not been used widely, likely because many cytokinesis proteins are essential. CRISPR-Cas9 was used to create non-muscle myosin heavy chain II isoform A and B (NMIIA and NMIIB; referring to the heavy chain encoded by *MYH9* and *MYH10*) loss-of-function alleles and determine their relative contributions to cytokinesis in WI-38 VA13 cells (immortalized human lung fibroblasts; Yamamoto et al., 2019). NMIIB was more strongly enriched in the ring compared to NMIIA, and excess NMIIB displaced most of NMIIA from the ring (Yamamoto et al., 2019). In contrast, NMIIA controlled cortical stiffness (Yamamoto et al., 2019). The loss of NMIIA or NMIIB caused different changes in ring ingression speed, and the authors proposed that the proper balance of NMIIA and NMIIB is required to control the length of equatorial actomyosin filaments (Yamamoto et al., 2019). Another study used CRISPR-Cas9 in HeLa cells to generate loss-of-function alleles of MICAL3, a MICAL-family protein (Liu et al., 2016). They used these alleles to show that MICAL3 regulates abscission and is recruited to the central spindle by MKLP1. Rescue with exogenous constructs lacking different domains of MICAL3 suggested that its role in abscission is independent of its F-actin monooxygenase activity (Liu et al., 2016). CRISPR-Cas9-mediated loss-of-function studies also revealed a surprising role for chloride intracellular channel 4 (CLIC4) in cytokinesis in HeLa cells (Uretmen Kagiali et al., 2019). CLIC4 localized to the midbody, and loss-of-function mutations caused a small increase in the proportion of cells that failed cytokinesis (Uretmen Kagiali et al., 2019). Cells showed abnormal cortical blebbing and changes in the levels of phosphorylated ezrin, suggesting a role for CLIC4 in regulating the cortical actin network (Peterman et al., 2020; Uretmen Kagiali et al., 2019).

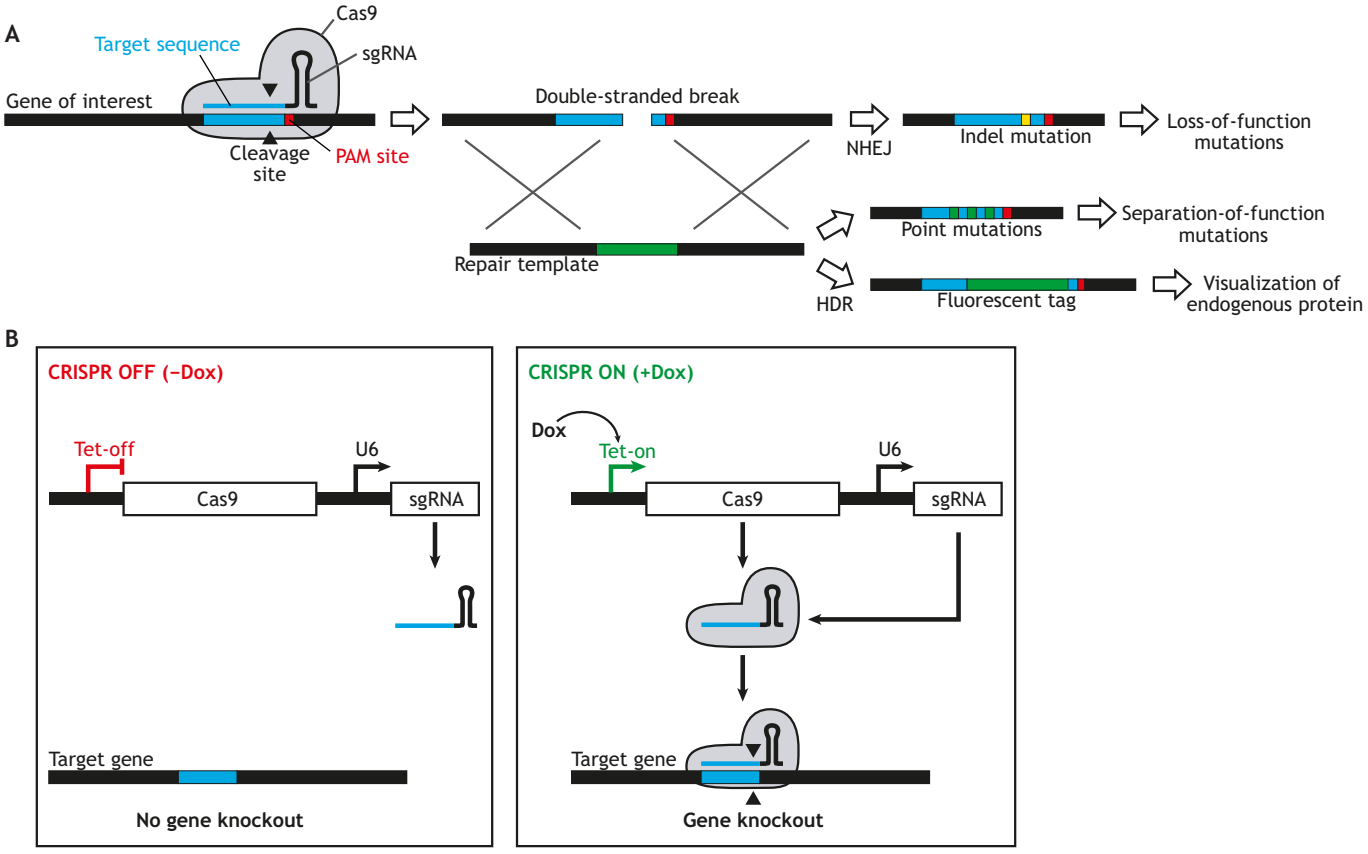

Fig. 2. Mechanism of CRISPR-Cas9 gene editing. (A) A schematic showing CRISPR-Cas9 gene editing through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Cas9 cleavage is directed by the sgRNA at a target site (blue) located adjacent to a PAM site (red) and results in a double-stranded break (DSB). NHEJ can introduce indel mutations (yellow) at the DSB site. HDR requires a repair template, which can be provided to introduce mutations or fluorescent tags (green) into the genome. (B) Schematics showing a doxycycline (Dox)-inducible CRISPR-Cas9 knockout system. This system consists of a Dox-inducible Cas9 and an sgRNA expressed from a constitutive promoter (e.g. U6 promoter). The addition of Dox (+Dox) induces Cas9 expression, which leads to the generation of indel mutations.

The use of CRISPR-Cas tools can help overcome some of the limitations of RNAi, but it also presents unique challenges. CRISPR-Cas9 generates stable and penetrant loss-of-function mutations, whereas RNAi is transient and variable. However, CRISPR-Cas9 relies on transfection or microinjection and is dependent on the efficiency of Cas9-sgRNA targeting and DNA repair. Loss-of-function alleles must be sequenced, and loss of protein expression must be verified, as is done for RNAi. In contrast to RNAi, this is done to verify that frameshift mutations lead to a functional knockout. Indeed, it has been shown that frameshift mutations can be rescued by exon skipping or translation re-initiation, which can restore protein production (Sharpe and Cooper, 2017; Smits et al., 2019; Zhang, 2019). Although less efficient, using two sgRNAs to delete essential exons by NHEJ is preferable to introducing indels (Chen et al., 2014; Swider et al., 2019). This approach has been shown to efficiently generate accurate gene deletions, which can be screened easily by PCR (Guo et al., 2018). Targeted gene deletions can also be generated by HDR, and a gene can be deleted and replaced with a selection marker or a series of stop codons (Wang et al., 2018; Au et al., 2019). However, this approach can only be used in organisms where edits are efficiently introduced by HDR (e.g. *C. elegans*). Importantly, strains or cell lines carrying homozygous loss-of-function mutations in essential genes cannot be generated, and RNAi is still preferred to study genes that are essential for cytokinesis.

Conditional loss-of-function mutations can be used to study essential genes, because they do not affect viability during routine maintenance. Conditional alleles were previously generated by random mutagenesis (e.g. O'Connell et al., 1998), but CRISPR-Cas9 offers a way to generate conditional loss-of-function mutations more directly. Inducible loss-of-function mutations can be triggered by introducing an inducible Cas9 gene along with a specific sgRNA (McKinley, 2018). Cas9 expression is induced chemically (e.g. using doxycycline) to generate loss-of-function mutations with high frequency (Fig. 2B). This design was used to probe the phenotypes caused by loss-of-function for 209 cell cycle genes in HeLa cells (McKinley and Cheeseman, 2017). These genes included well-known cytokinesis regulators such as Aurora B kinase, anillin and Ect2, and the loss-of-function cells showed the expected multinucleated phenotypes, thus proving the usefulness of this approach. As an alternative, transiently transfecting an sgRNA in cells stably expressing Cas9 can efficiently generate a high proportion of cells with loss-of-function alleles (Fig. 3A; Su et al., 2018). CRISPR-Cas9 can also be used to introduce recombination sites flanking a gene of interest by HDR, and knockouts can be induced by expressing a site-specific recombinase (Fig. 3A; Economides et al., 2013; Andersson-Rolf et al., 2017). The timing of knockout induction is crucial, as the appearance of loss-of-function phenotypes can be delayed (McKinley, 2018), and phenotypes must be assessed within one generation after knockout for essential genes. In organisms, cell-specific loss-of-function can be achieved by expressing Cas9 (or a recombinase if using the recombination system) under a cell-specific promoter, along with a constitutively expressed sgRNA (Fig. 3A; Chai et al., 2017; Fielmich et al., 2018). This approach was used in *C. elegans* to generate lineage-specific loss-of-function alleles of *ani-1* to study its role in the migration of Q cells, which require ANI-1 for cytokinesis (Fotopoulos et al., 2013; Chai et al., 2017). Importantly, in order to achieve efficient conditional knockouts, sgRNAs with high targeting efficiency must be selected (McKinley, 2018).

Using separation-of-function mutations to study cytokinesis proteins

Separation-of-function mutations are crucial to study protein function, interactions and regulation. Mutations are designed based on biochemical and structural data, and CRISPR-Cas9 can be used to introduce them endogenously via HDR (Fig. 2A; Verma et al., 2017). This approach has been primarily used to generate targeted mutations in cytokinesis genes in *C. elegans*, including in *ect-2* and *cyk-4* (Zhang and Glotzer, 2015), *nmy-2* (Osório et al., 2019) and *zen-4* (Basant et al., 2015). The introduction of an ECT-2 gain-of-function mutation and a CYK-4 GAP-inactivating mutation in the same *C. elegans* strain demonstrated that the main function of CYK-4 is to regulate ECT-2 activity for cytokinesis (Zhang and Glotzer, 2015). Similarly, mutations that impair the motor activity of NMY-2 (the worm homolog of the non-muscle myosin II motor heavy chain) without affecting its ability to cross-link F-actin were generated using CRISPR-Cas9 (Osório et al., 2019). By separating these functions, the authors showed that NMY-2 activity is required for ring assembly and constriction. Finally, CRISPR-Cas9-induced mutations in the S682 phosphorylation site of ZEN-4 were shown to phenocopy the effect of PAR-5 depletion (Basant et al., 2015). These results support a model where MKLP1 phosphorylation by Aurora B kinase relieves 14-3-3 inhibition to promote cortical contractility.

To become more widely applicable, CRISPR-Cas tools need to overcome the low efficiency of precise repair by HDR. Although some approaches can improve this efficiency (Liu et al., 2019), further improvements will make it more accessible to researchers in various fields. When introducing multiple mutations, a gene replacement approach can be used, where a mutant gene carrying the desired mutations is generated *in vitro* and inserted in place of the endogenous gene (Fig. 3B,C; Dickinson and Goldstein, 2016). This approach can be useful to introduce mutations that span multiple exons, or to replace an entire gene when a specific site cannot be targeted by Cas9 (Fig. 3C). However, only viable mutations can be introduced endogenously, restricting their use to studying non-essential functions. In *C. elegans*, mutations in essential genes can be rescued by an exogenous construct that can be removed by RNAi to allow the study of cytokinesis in the homozygous mutant embryos (Fig. 3B; Dickinson and Goldstein, 2016; Portegijs et al., 2016). For example, the motor-dead *nmy-2* alleles described earlier were generated as heterozygotes, and crossed with a strain expressing an exogenous wild-type *nmy-2* gene (Osório et al., 2019). The homozygous motor-dead mutant strain could be generated because it was rescued by the expression of exogenous wild-type NMY-2 (Osório et al., 2019). The wild-type protein was then depleted using RNAi, to demonstrate that the motor is required for ring assembly and constriction (Osório et al., 2019). Although arduous, the introduction of mutations at the endogenous locus can reveal more reliable phenotypes, since the mutants are expressed at endogenous levels (Portegijs et al., 2016).

Modulating gene expression with CRISPRi and CRISPRa

Other CRISPR-Cas-based tools allow for the control of gene expression (Shakirova et al., 2020). CRISPRi makes use of a nuclease-dead Cas9 (dCas9, which can bind to its target but is unable to cleave DNA) fused to a transcriptional repressor and targeted to a promoter region by an sgRNA to knock down a target gene (Fig. 4A; Gilbert et al., 2013; Qi et al., 2013; Mandegar et al., 2016; Shakirova et al., 2020). CRISPRa uses dCas9 fused to a transcriptional activator and targeted to a promoter region by an sgRNA to upregulate gene expression (Fig. 4B; Gilbert et al., 2013; Maeder et al., 2013; Kleinjan et al., 2017; Shakirova et al., 2020). Inducible CRISPRi systems use various inducers to activate the expression of the dCas9–repressor fusion, and precise knockdown can be achieved by fine-tuning the strength or time of induction

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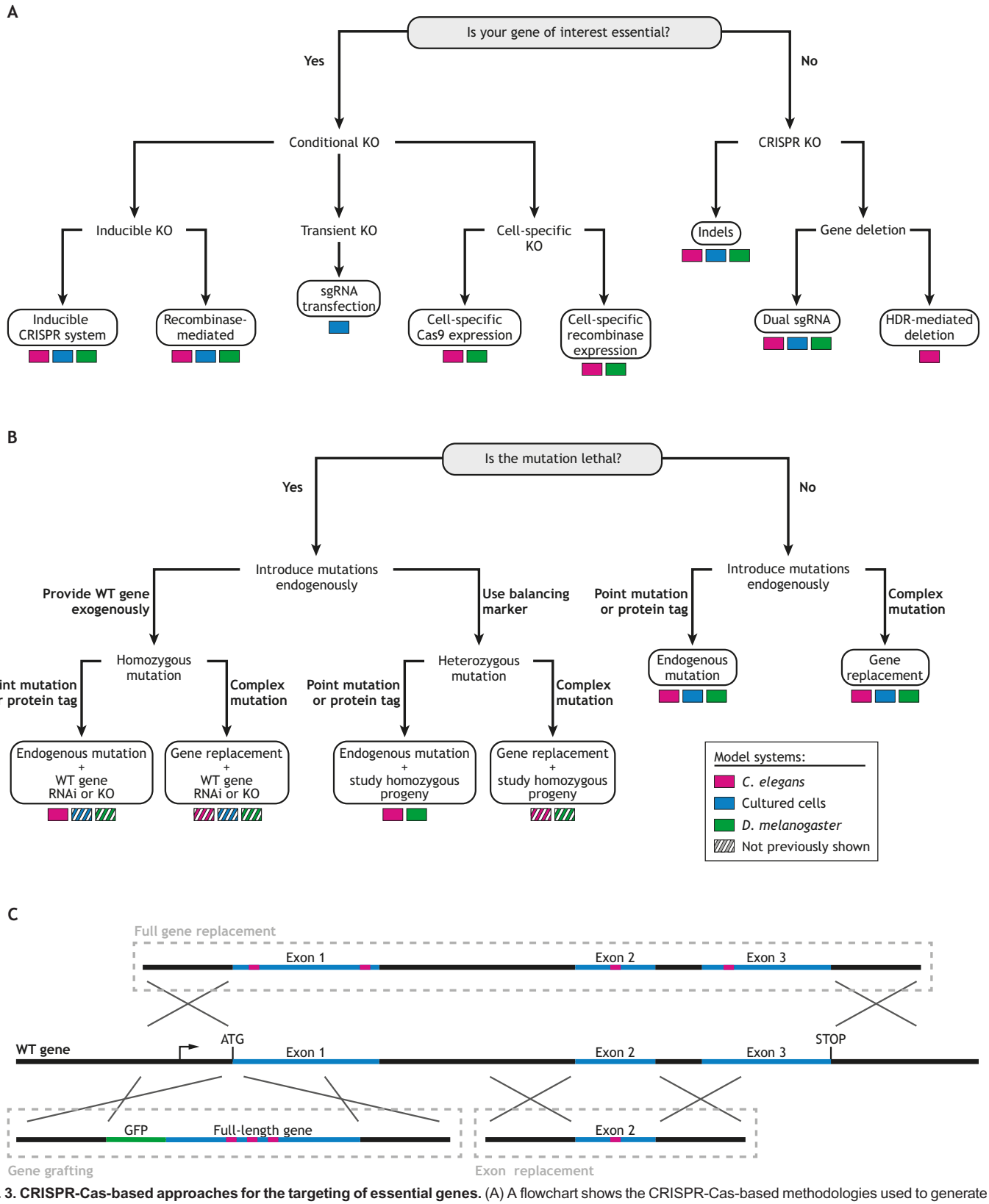

Fig. 3. CRISPR-Cas-based approaches for the targeting of essential genes. (A) A flowchart shows the CRISPR-Cas-based methodologies used to generate knockouts (KOs) in essential (left) and non-essential genes (right). The methodologies described for essential genes can also be used to target non-essential genes. (B) A flowchart shows the CRISPR-Cas-based approaches to introduce lethal (left) and viable (right) mutations endogenously. Colored rectangles indicate the model systems that apply to each strategy (cultured cells in blue, *C. elegans* in pink and *D. melanogaster* in green). Rectangles with hatched colors indicate the strategies that could be used in a model system, but that have not yet been established experimentally. (C) A schematic shows the gene replacement strategy used to introduce endogenous mutations. The gene locus can be replaced with a mutant sequence generated *in vitro* (gene replacement; top). A single exon can also be replaced with an exon mutated *in vitro* (exon replacement; bottom right). Alternatively, a full coding sequence carrying the desired mutations can be inserted downstream of the endogenous promoter (gene grafting; bottom left).

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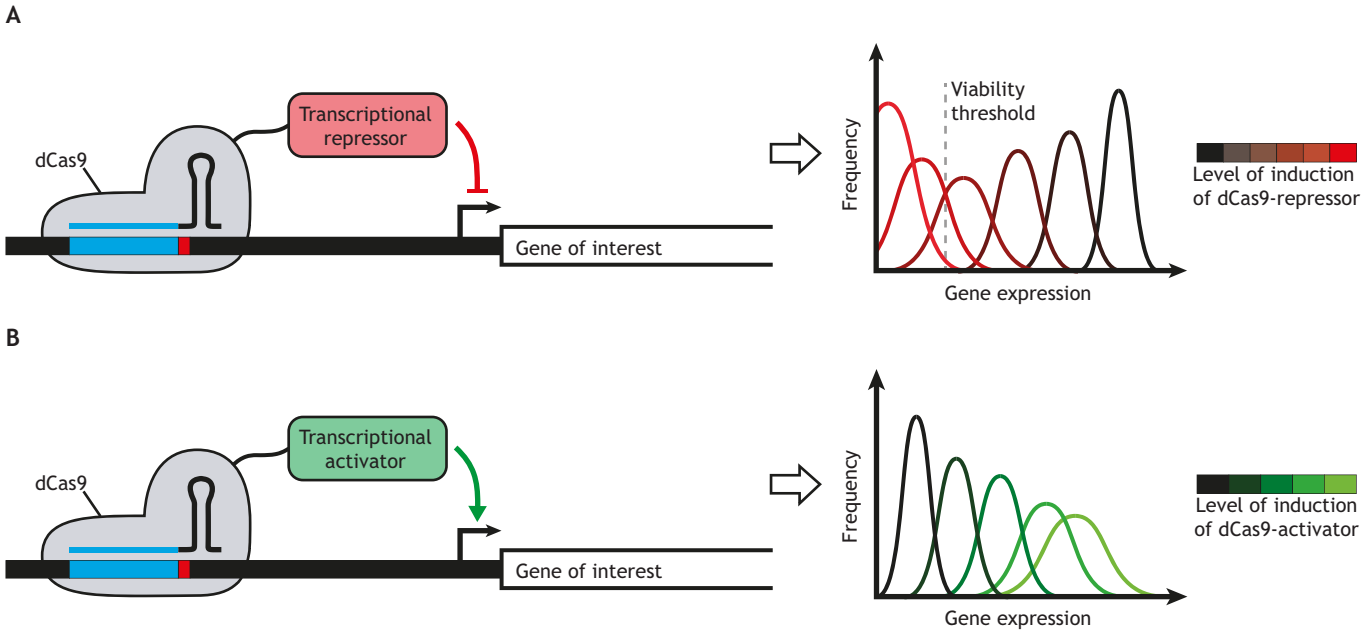

Fig. 4. Modulating gene expression with CRISPRi and CRISPRa. (A) A schematic showing the repression of gene expression using CRISPRi. A nuclease-dead Cas9 (dCas9) fused to a transcriptional repressor is targeted to a promoter region using an sgRNA to downregulate gene expression (left). The hypothetical graph shows the strength of knockdown that can be obtained by fine-tuning CRISPRi induction, indicated as the frequency of cells with different protein levels (right). This can be achieved by optimizing the strength (e.g. inducer concentration) or time of induction to obtain a precise level of knockdown. (B) A schematic shows gene activation using CRISPRa. A dCas9-transcriptional activator fusion is used to upregulate the expression of the target gene (left). The hypothetical graph shows the expression levels that can be obtained by fine-tuning the induction of CRISPRa, shown as the frequency of cells with different protein levels (right).

(Fig. 4A; Larson et al., 2013; Mandegar et al., 2016; Kleinjan et al., 2017; Pickar-Oliver and Gersbach, 2019; Gamboa et al., 2020). More recently, an artificial neural network was trained to predict the CRISPRi activity of sgRNAs that carry mismatches compared to their target sequence (Jost et al., 2020). This predictive model can be used to find candidate sgRNAs designed to achieve a specific level of knockdown. Combined with endogenous fluorescent reporters to quantify gene expression, these approaches could be used to: (1) titrate gene expression to a precise level, (2) attribute specific phenotypes to a precise window of gene expression, and (3) to investigate the mechanism underlying an intermediate phenotype. Mild phenotypes generated using weak loss-of-function, temperature-sensitive alleles or weak RNAi have been used to build models of cytokinesis protein function in *C. elegans* (Velarde et al., 2007; Budirahardja and Gönczy, 2008; Jordan et al., 2016; Zhuravlev et al., 2017; Chan et al., 2019). CRISPRi could be used to controllably induce mild phenotypes in cultured cells to help refine current models of cytokinesis. Moreover, growing evidence shows that the regulation of cytokinesis varies during development (Fotopoulos et al., 2013; Li et al., 2016; Davies et al., 2018). Precise control over gene expression could help determine the exact protein levels required for cytokinesis in different cellular contexts. Although it requires expressing multiple components, CRISPRi could be more tunable, more stable and present fewer off-target effects than RNAi (Stojic et al., 2018).

Using CRISPR-Cas tools to generate imaging tools to study cytokinesis

CRISPR-Cas9 can also be used to introduce fluorescent protein tags endogenously to monitor the localization of cytokinesis proteins. This tool is particularly useful for cytokinesis studies in *C. elegans* and mammalian cells, but less common in *D. melanogaster*, where other recombination-based approaches are typically used. Fluorescent tags are inserted into a target locus by HDR (Fig. 2A; Bukhari and Müller, 2019). NHEJ repair can provide higher efficiency than HDR, but this method presents different challenges (Banan, 2020). For example, whereas HDR can introduce insertions away from the Cas9 cleavage site, depending on the sequence of the homology arms, NHEJ-based insertions can only occur at the free DNA ends generated by Cas9.

The use of endogenous tags provides a more accurate picture of protein levels, post-translational modifications and interactions, all of which can influence localization and function. For examples, the endogenous tagging of Aurora B kinase in HeLa cells provides more reliable expression and localization compared to transgenic expression (Mahen et al., 2014). Unfortunately, few cytokinesis proteins have been tagged endogenously in mammalian cells (Mahen et al., 2014; Cai et al., 2018; Mann and Wadsworth, 2018; Hoffman et al., 2019). However, this approach has been used to visualize several cytokinesis proteins in *C. elegans*, including UNC-59 (the homolog of septin 7; Chen et al., 2019), AIR-2 (the worm Aurora B kinase; Bai et al., 2020), PLST-1 (plastin 1) (Ding et al., 2017), CYK-1 [the homolog of diaphanous-related formin 1 (mDia1); Davies et al., 2018], LET-502 (the worm homolog of the ROCK proteins; Bell et al., 2020) and NMY-2 (Dickinson et al., 2013). PLST-1 is a component of the contractile ring and was shown to function as an actin crosslinker that increases cortical connectivity (Ding et al., 2017). In another study, the endogenous tagging of CYK-1 helped demonstrate a different requirement for CYK-1 in endomesodermal (EMS) and P_2 cells compared to ABa and ABp cells in the four-cell *C. elegans* embryo (Davies et al., 2018). Endogenous tagging of the RHO-1 effector LET-502 revealed that germinal center kinase 1 (GCK-1, the homolog of STK24, STK25 and STK26) and cerebral cavernous malformation 3

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(CCM-3; the homolog of PDCD10) dampen contractility by negatively regulating active RHO-1 (Bell et al., 2020). NMY-2 was one of the first proteins to be tagged using CRISPR-Cas9 in *C. elegans* (shown in Fig. 5A; Dickinson et al., 2013), paving the way for numerous studies of the mechanisms regulating cytokinesis and other contractile events during embryogenesis (Wernike et al., 2016; Singh et al., 2019). Endogenous tags can be used to measure protein localization during cytokinesis (Fig. 5B), quantify contractile ring components (Fig. 5C), and to correlate cytokinesis phenotypes with precise protein levels after knockdown (Fig. 5D).

Cellular markers have been useful to study cytokinesis. These include probes that localize to the plasma membrane [e.g. the PH domain of phospholipase Cγ (PLCγ)], microtubules (e.g. fluorescent-tagged tubulin or SiR-tubulin), F-actin (e.g. fluorescent-tagged LifeAct or SiR-actin) or chromatin (e.g. Hoechst or fluorescent-tagged histones). As described above, the GFP-tagged NMY-2 *C. elegans* strain (Fig. 5A; Dickinson et al., 2013) has been an invaluable tool to study cytokinesis parameters such as equatorial cortical localization, ingression speed and ring closure symmetry (Carvalho et al., 2009; Khaliullin et al., 2018; Osório et al., 2019). Although most human proteins have yet to be tagged endogenously, the tools to generate various endogenous tags in human cells already exist. These tools include those to generate mEGFP–TUBA1B (α-tubulin), mEGFP–ACTB (β-actin) or mEGFP–MYH10 (non-muscle myosin IIB heavy chain), which have been tested on human iPSCs (induced pluripotent stem cells; Roberts et al., 2017). The Allen Institute for Cell Science has continued to add to these tools (https://www.allencell.org/), which can be used to generate the same endogenous tags in other human cell lines.

It is important to be aware of the limitations associated with using endogenous tags to study protein function. Tagging efficiency is generally low and requires extensive screening to obtain homozygous cell lines or strains (Banan, 2020). This can be overcome by using a self-complementing split fluorescent protein, which fluoresces when a small (16 amino acids) and large (213 amino acids) fragment complement each other (Kamiyama et al., 2016; Leonetti et al., 2016;

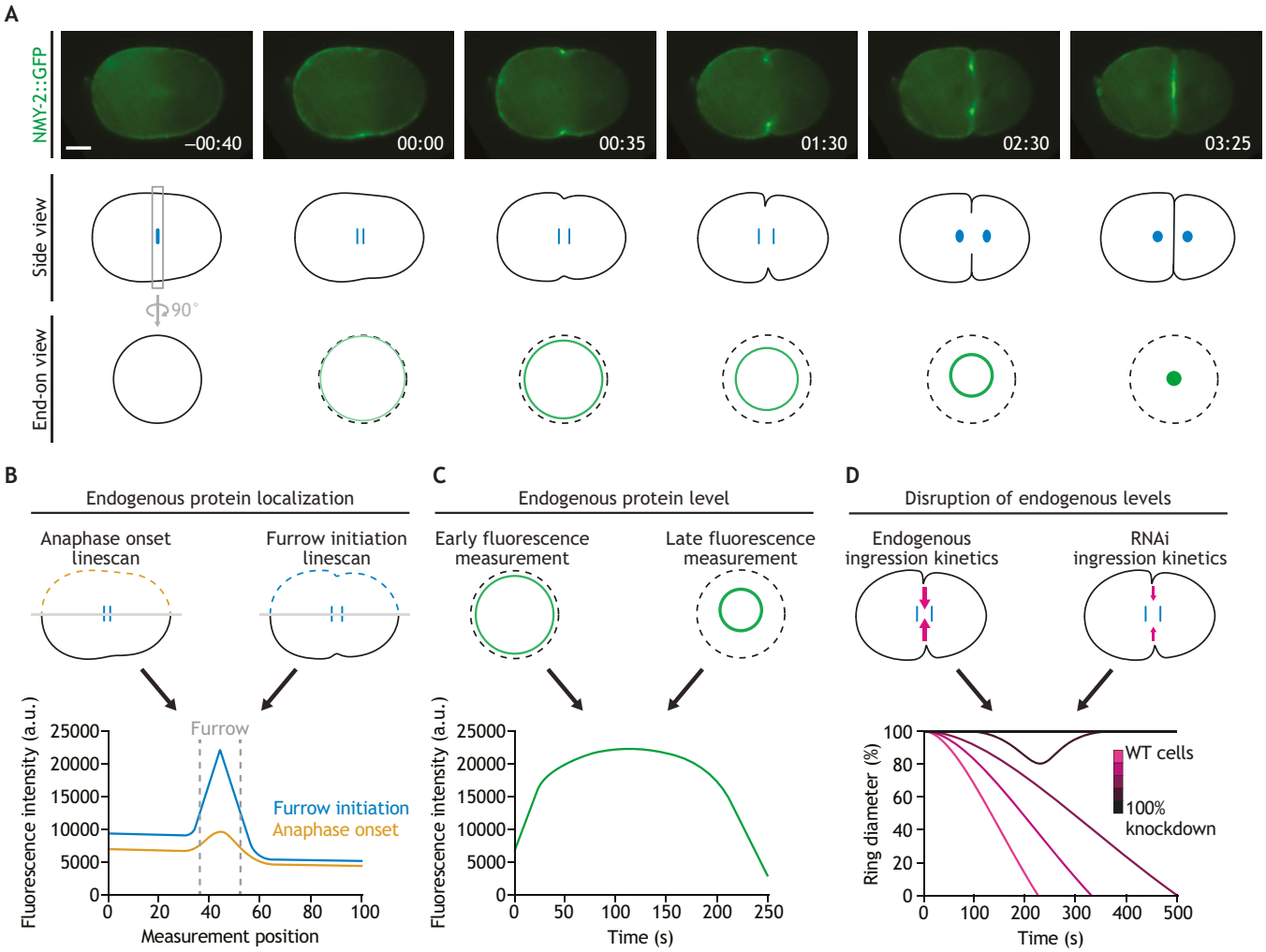

Fig. 5. Uses of endogenous tags to study cytokinesis. (A) Time-lapse images show a one-cell *C. elegans* embryo expressing endogenously tagged NMY-2 during cytokinesis (times are in minutes:seconds with 00:00 being anaphase onset). Images are courtesy of Imge Özügergin (Department of Biology, Concordia University, Montreal, QC, Canada). Scale bar: 10 µm. The cartoon underneath shows the progression of cytokinesis after anaphase onset from both a side (middle panel, DNA is shown in blue) and end-on view (bottom panel). (B) The localization of cortical NMY-2 can be analyzed using a linescan to measure fluorescence intensity along the anterior-posterior axis at different time points. As shown in the mock graph (a.u., arbitrary units), the linescan data can be used to show the change in fluorescence intensity in the furrow over time. (C) Total levels of endogenous protein in the ring can be measured at different time points, and plotted to show how these levels correlate with different stages of ring ingression. (D) Ring closure kinetics can be measured and precisely correlated with endogenous protein levels after RNAi or other perturbations to determine threshold requirements. WT, wild type.

Feng et al., 2017). The shorter fragment can be used to tag various proteins, whereas the larger fragment is expressed constitutively. This increases the efficiency and throughput of endogenous tagging, and has been done for multiple proteins, including histone 2B, β-actin and β-tubulin (Leonetti et al., 2016). This strategy also allows for the tissue-specific visualization of endogenous proteins by expressing the large fragment under a tissue-specific promoter (Cheerambathur et al., 2019). Another challenge is the screening and visualization of weakly expressed proteins. In some cases, adding tandem repeats of the fluorescent protein or split protein tag can enhance the signal (Dambournet et al., 2014; Leonetti et al., 2016; Pinheiro et al., 2017). Protein tags can also affect the folding, stability and dynamics of the tagged protein or its ability to assemble into complexes (Koch et al., 2018; Shaw et al., 2020). This can be alleviated by using protein linkers or smaller probes (Kamiyama et al., 2016; Feng et al., 2017). Despite these concerns, endogenous tagging with fluorescent markers will enable new discoveries in the field of cytokinesis.

Future perspectives

Our knowledge of cytokinesis is restricted to a small subset of cell types, yet growing evidence suggests that the mechanisms regulating cytokinesis vary between different cell types and in the context of tissues. For example, studies of symmetrically dividing epidermal cells in *Drosophila* embryos have revealed that cytokinesis must be coordinated with junction remodeling (Guillot and Lecuit, 2013; Herszterg et al., 2013; Pinheiro et al., 2017). In the four-cell *C. elegans* embryo, cells have different formin-dependent F-actin requirements, which is regulated intrinsically or extrinsically depending on the cell type (Davies et al., 2018). During *C. elegans* mid-embryogenesis, neuronal precursor cells uniquely express and require ANI-1 for cytokinesis (Fotopoulos et al., 2013). Gene editing tools could facilitate studies of cytokinesis in more diverse cell types, including patient-derived cells. CRISPR-Cas9 was used to link mutations in the citron kinase gene to cytokinesis failure in neural progenitor cells derived from microcephalic patient iPSCs (Li et al., 2016). Recently, CRISPR-Cas9 permitted the endogenous tagging of proteins to monitor the mitotic spindle in human liver organoids (Artegiani et al., 2020). Gene-editing tools can overcome the limitations associated with RNAi and transgene expression, and extend our understanding of cytokinesis and its conservation to a diversity of animal models, including non-model species useful for studies of evolution and adaptation.

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Competing interests

The authors declare no competing or financial interests.

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