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Sumoylation controls retinal progenitor proliferation by repressing cell cycle exit in *Xenopus laevis*

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ABSTRACT

Precisely controlled progenitor proliferation is essential for normal development. However, molecular mechanisms, which control the correct timing of cell cycle withdrawal during development, have been poorly understood. We show here that ubc9, a sumo-conjugating enzyme, controls the cell cycle exit of retinal progenitors. We found that *ubc9* is highly expressed in retinal progenitors and stem cells in *Xenopus* embryos. Ubc9 physically and functionally associates with *Xenopus* hmgb3, which is required for retinal cell proliferation, and prolonged expression of ubc9 and hmgb3 results in suppression of the cell cycle exit of retinal progenitors in a sumoylation-dependent manner. Overexpression of *ubc9* and *hmgb3* decreased expression of the cell-cycle inhibitor $p27^{Xic1}$. Furthermore, progenitor proliferation is regulated, at least in part, by sumoylation of transcription factor Sp1. These results suggest a significant role of sumoylation for cell cycle regulation in retinal progenitors.

Introduction

The vertebrate neural retina is a complex sensory tissue whose function depends on the production of a sufficient number of cells of each retinal cell type and the correct formation of laminar cytoarchitecture. The seven major cell types that compose the neural retina arise from a pool of multipotent progenitors (Young, 1985; Cepko et al., 1996). Birthdating studies have demonstrated that the seven retinal cell types are generated in an evolutionarily conserved order during development, although multiple cell types are simultaneously produced at any given developmental stage (Cayouette et al., 2006; Cepko et al., 1996). Therefore, retinal progenitor cells stop proliferation to differentiate into the cell types that are generated earlier, but also proliferate extensively at an appropriate rate to produce a sufficient number of later cell types. Thus, cell proliferation and cell differentiation occur in parallel in the developing vertebrate retina (Cepko et al., 1996; Donovan and Dyer, 2005; Harris, 2009; Levine and Green, 2004; Young, 1985). A number of extrinsic cues, including Wnt, Hedgehog and Notch signaling pathways have been implicated in the control of retinal progenitor proliferation and differentiation in the developing retina (Denayer et al., 2008; Dorsky et al., 1997; Van Raay et al., 2005; Wallace, 2008). On the other hand, the significance of the cell-intrinsic program has been suggested by the clonal density culture of retinal progenitor cells from the rat retina at embryonic day 17.5 (E17.5) (Cayouette et al., 2003). A number of homeodomain transcription factors have been implicated in the control of retinal progenitor proliferation, including *rax*/*Rx*, *Pax6*, *Chx10*, *Six3*, *six6*/*optx2* and *Prox1*. They are involved in the regulation of cell fate competency, cell proliferation and cell differentiation of retinal progenitors (Burmeister et al., 1996; Casarosa et al., 2003; Dyer et al., 2003; Furukawa et al., 2000; Green et al., 2003; Li et al., 2002; Marquardt et al., 2001; Zuber et al., 1999). However, molecular mechanisms controlling the balance of two opposing cell behaviors, cell proliferation and cell cycle exit during development, remain to be elucidated.

Cyclin-dependent kinase inhibitors (CKI) play a significant role in the regulation of the cell cycle. Two families of Cdk inhibitors, the Cip/Kip family, which includes $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$, and the Ink4 family, comprised of $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, have been identified. CKI can inhibit the various cyclin-Cdk complexes that control G1 progression and entry into the S phase, and its overexpression in various cell lines arrests the cell cycle in G1 (Besson et al., 2008; Sherr and Roberts, 1999). In mice $p27^{Kip1}$ regulates the cell cycle withdrawal of late retinal progenitor cells, and in *Xenopus* $p27^{Xic1}$ regulates cell cycle exit and Müller glia development in the developing retina (Levine et al., 2000; Ohnuma et al., 1999).

Sumoylation is a highly conserved evolutionary pathway, from yeast to vertebrates. Ubc9 is the only known small ubiquitin-related modifier (sumo) E2-conjugating enzyme. In *Saccharomyces cerevisiae*, *ubc9* depletion results in cell cycle arrest at the G2/M phase (Seufert et al., 1995). By contrast, in *Schizosaccharomyces pombe*, the *hus5*

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(ubc9 homolog) deletion mutant is viable, however, the cells exhibit high levels of abortive mitosis and chromosome missegregation (al-Khodairy et al., 1995). RNAi-mediated knockdown of *ubc9* in *Caenorhabditis elegans* results in embryonic arrest after gastrulation and pleiotropic defects in larval development such as vulval eversion at the fourth larval stage and abnormal tail morphology (Jones et al., 2002). In *Drosophila melanogaster*, the *ubc9* lethal mutant, known as *lesswright*, dominantly suppresses the nondisjunction and cytological defects of female meiotic mutations that affect spindle formation (Apionishev et al., 2001). In zebrafish, ubc9 regulates mitosis and cell survival during development. Loss of *ubc9* leads to compromised mitosis and cellular overgrowth, and *ubc9* is required for tissue-specific cell viability in zebrafish (Nowak and Hammerschmidt, 2006). In mice, the ubc9-mediated sumo pathway is essential for nuclear integrity and chromosome segregation (Nacerddine et al., 2005). Sumo post-translationally modifies many proteins with roles in diverse processes including regulation of transcription, chromatin structure, DNA repair, protein localization, and protein activity (Geiss-Friedlander and Melchior, 2007; Hay, 2005; Johnson, 2004). It was reported that neural crest precursor formation is regulated by sumoylation of SoxE in *Xenopus*, resulting in a change in the physiological role of the SoxE protein (Taylor and Labonne, 2005). Recently, it was also reported that sumoylation has an important role in retinal photoreceptor differentiation (Onishi et al., 2009). However, the exact role of *ubc9* in retinal development remains to be elucidated.

Rax/Rx is a transcription factor that is predominantly expressed in retinal progenitors and is essential for eye development in vertebrates (Furukawa et al., 1997; Mathers et al., 1997). *Rax* is conserved among species, including human, mouse, chicken, fish and *Xenopus*. We previously demonstrated that *Xenopus* high mobility group box 3 (*hmgb3*) is one of the downstream factors of rax (Terada et al., 2006). Overexpression and loss-of-function experiments have indicated that *hmgb3* has a significant role in retinal progenitor proliferation during eye development in *Xenopus*. Hmgb3 is an evolutionally conserved nuclear protein without a transactivation domain and has been thought to function as a chromatin modulator, suggesting that hmgb3 functions through a protein complex. In the current study, we identified ubc9, a sumo conjugating enzyme, to be an interacting partner of hmgb3. We demonstrated that ubc9 regulates retinal progenitor proliferation in a hmgb3-dependent manner, at least in part, via the suppression of $p27^{Xic1}$ expression through Sp1 sumoylation. Our results suggest that protein sumoylation plays an important role in controlling retinal progenitor proliferation.

Materials and methods

Yeast two-hybrid assays

We carried out a yeast two-hybrid screen using MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The *AH109* yeast strain bearing the bait plasmid *pGBKT7-hmgb3* was transformed with the mouse retinal (Postnatal day 0 (P0)-P3) cDNA library in a *pGADT7* vector. Initially, about 400,000 clones were screened. Yeast colonies that grew at 30 °C on SD-Trp-Leu-His were re-streaked on SD-Trp-Leu-His-Ade (218 colonies). Streaked yeast colonies that grew on SD-Trp-Leu-His-Ade (121 colonies) were subjected to the β-galactosidase (β-gal) assay. 24 colonies were positive in the β-gal assay. Rescued plasmids from yeast into *Escherichia coli* strain *DH5α* were classified by enzyme digestion pattern. Each representative plasmid was re-introduced into the yeast strain *AH109* together with *pGBKT7-hmgb3* to confirm the reproducibility and specificity of interactions. Seven clones were obtained and six of them were *ubc9* cDNAs. The amino acid sequences of mouse ubc9 and *Xenopus* ubc9 are identical. Therefore, the mouse *ubc9* cDNA was used for overexpression experiments using *Xenopus* embryos.

Embryo manipulations

Embryos were obtained from *Xenopus laevis* adult frog by hormone-induced egg laying and *in vitro* fertilization. Embryos were staged according to Nieuwkoop and Faber (1994). Glucocorticoid receptor fusion proteins were activated by adding 25 μM dexamethasone (DEX) (Sigma, D1756) into the culture medium. Injected embryos were raised at 15 °C. *Xenopus* embryos were abdominally injected with 5-Bromo-2'-deoxy-uridine (BrdU) (20 mM) for BrdU labeling.

RNA synthesis and RNA microinjection

Capped RNAs were synthesized using the MESSAGE mMACHINE kit (Ambion). One animal dorsal blastomere was injected for phenotype observations at the 8-cell stage. The amount of injected synthetic RNAs were as follows; 75 pg of *lacZ*, 100 pg of *EGFP*, 225 pg of *hmgb3*, 250 pg of *hmgb3* (*mut*), 100 pg of *ubc9*, 200–800 pg of *ubc9* (*C93A*), 200 pg or 400 pg of *sumo-Sp1-GR*, 800 pg of *Sp1(17-723)-GR*, 400 pg of *Sp1-GR*.

X-gal staining and in situ hybridization

Embryos were grown to a desired stage and fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgCl_2$, 3.7% formaldehyde) for 30 min and transferred into the X-gal staining solution until staining was apparent. *In situ* hybridization was performed essentially as described (Harland, 1991). RNA probes of *Xenopus hmgb3* and *Xenopus rax* were previously described (Terada et al., 2006).

Quantification of enlarged eye size

Embryos injected with synthetic RNA were grown to stage 37/38 and fixed in MEMFA followed by X-gal staining. Quantification of enlarged eye was performed as previously described (Terada et al., 2006). The eye diameters were measured by Image J and used to calculate the percent increase in eye diameter on the injected side to the un-injected side. The epidermis overlaying the eye was removed.

Animal cap dissections

Animal caps were dissected from stage 8/9 embryos in 0.5x MMR (Marc's Modified Ringer's solution). The caps were incubated until sibling embryos reached stage 19/20 and then subjected to further analysis.

Immunohistochemistry

Immunostaining of frozen sections was performed essentially as described previously with minor modifications (Terada et al., 2006). A blocking solution containing 0.2% of TritonX-100 was used. Sections were incubated for 1 h in 2N HCl prior to BrdU immunodetection. For immunostaining, anti-β-galactosidase (β-gal) antibody (Promega, #Z3781), anti-phospho-histone H3 antibody (Upstate, #06-570), anti-BrdU antibody (Fitzgerald, #20-BS17), anti-calbindin (calbiochem #PC253L), anti-islet1 (DSHB, clone 39.4D5), and anti-PCNA antibody (DAKO, M0879) were used for primary antibodies, Cy3-conjugated-anti-rabbit IgG (Jacson ImmunoResearch Laboratories, #711-165-152), Cy3-conjugated-anti-mouse IgG (Jacson ImmunoResearch Laboratories, #711-165-150), Alexa Fluor 488-conjugated-anti-sheep IgG (Invitrogen, #A11015) and Alexa Fluor 488-conjugated-anti-mouse IgG (Invitrogen, #A11001) were used for secondary antibodies. For immunoprecipitation and Western blots, anti-HA antibody (Santa Cruz, #sc-805), anti-myc antibody (Santa Cruz, #sc-40), anti-FLAG antibody (M2) (Sigma, #F3165) were used.