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Loss of histone deacetylase HDAC1 induces cell death in *Drosophila* epithelial cells through JNK and Hippo signaling

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ABSTRACT

Inactivation of HDAC1 and its homolog HDAC2 or addition of HDAC inhibitors in mammalian systems induces apoptosis, cell cycle arrest, and developmental defects. Although these phenotypes have been extensively characterized, the precise underlying mechanisms remain unclear, particularly in *in vivo* settings. In this study, we show that inactivation of Rpd3, the only HDAC1 and HDAC2 ortholog in *Drosophila*, induced apoptosis and clone elimination in the developing eye and wing imaginal discs. Depletion of *Rpd3* by RNAi cell-autonomously increased JNK activities and decreased activities of Yki, the nuclear effecter of Hippo signaling pathway. In addition, inhibition of JNK activities largely rescued *Rpd3 RNAi*-induced apoptosis, but did not affect its inhibition of Yki activities. Conversely, increasing the Yki activities largely rescued *Rpd3 RNAi*-induced apoptosis, but did not affect its induction of JNK activities. Furthermore, inactivation of Mi-2, a core component of the Rpd3-containing NuRD complex strongly induced JNK activities; while inactivation of Sin3A, a key component of the Rpd3-containing Sin3 complex, significantly inhibited Yki activities. Taken together, these results reveal that inactivation of Rpd3 independently regulates JNK and Yki activities and that both Hippo and JNK signaling pathways contribute to *Rpd3 RNAi*-induced apoptosis.

1. Introduction

Histone deacetylases (HDACs) regulate transcription and other processes by removing acetyl modification from histones and other proteins (Kurdistani and Grunstein, 2003; Ng and Bird, 2000). Human or mouse has 18 HDACs that can be divided to four classes. Class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8. HDAC1 and HDAC2 have very similar protein sequences with redundant functions (Gregoretti et al., 2004; Moser et al., 2014). Loss of both HDAC1 and HDAC2 induces apoptosis, cell cycle arrest, and DNA damage in many cell types. Importantly, HDAC1 and HDAC2 specific inhibitors are potential anti-cancer drugs (Dokmanovic et al., 2007; Glozak and Seto, 2007; Johnstone, 2002; Kelly and Cowley, 2013; Minucci and Pelicci, 2006; Ropero and Esteller, 2007; West and Johnstone, 2014). However, the safety of the HDAC inhibitors is a significant concern because HDAC1 and HDAC2 also play important roles in regulating gene expression in development. Loss of HDAC1 and HDAC2 led to profound defects in tissues with epithelial structures, such as intestine and epidermis (Brunmeir et al., 2009; Gonneaud et al., 2015; Winter et al., 2013). Furthermore, the enzymatic activities of HDAC1 and HDAC2 are dependent on their incorporation into different protein complexes, such as the Sin3, NuRD and CoREST complexes, which bind and deacetylate distinct substrates (Kelly and Cowley, 2013; Moser et al., 2014). The specific roles of these different complexes in developing epithelial cells are not well understood.

Class I HDACs are highly conserved during evolution. Yeast has a single HDAC1, also known as Rpd3. *Drosophila* has two Class I HDACs: Rpd3 and HDAC3. Rpd3 plays important functions in regulating gene expression by interacting with transcriptional repressors such as Groucho, Polycomb and Atrophin (Chang et al., 2001; Chen et al., 1999; Tea et al., 2010; Tie et al., 2001; Zhang et al., 2013). In addition, loss of *Drosophila* Rpd3 inhibited cell growth and induced apoptosis (Zhu et al., 2008), suggesting that Rpd3 is required for cell survival similar to the roles of HDAC1 and HDAC2 in mammalian cells. However, the downstream pathways affected by loss of Rpd3 are largely unknown.

The key signaling pathways regulating cell proliferation, differentiation, and cell death are highly conserved between *Drosophila* and mammals. The well-characterized developmental programs in *Drosophila* combined with the sophisticated genetic, developmental, and cellular approaches make *Drosophila* a powerful model to investigate the functions of genes and pathways *in vivo*. *Drosophila* imaginal discs at larval stages have epithelial structures and most of cells keep proliferating, which make them as ideal models to study gene functions in proliferation, differentiation, and apoptosis (Hariharan and Bilder, 2006; Morata, 2001; Pan, 2007; Vidal and Cagan, 2006). In this study, we address how Rpd3 regulates cell survival by analyzing mosaic clones depleting *Rpd3* in *Drosophila* imaginal discs. We show that loss of Rpd3

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activates JNK signaling and inhibits Yki activities, and that both JNK and Yki contribute to *Rpd3 RNAi*-induced apoptosis and clone elimination. These results provide new insights into the roles of HDAC1 and HDAC2 *in vivo* and contribute to a better understanding on the potential effects of the Class I HDAC inhibitors on epithelial cells.

2. Materials and methods

2.1. Drosophila stocks

Fly stocks used in this study include: *Rpd3 RNAi* (BL 33725) (Bloomington Stock Number), *Rpd3 RNAi-2* (BL 34846), *UAS-Puc* (Martin-Blanco et al., 1998), *pucE69* (Riesgo-Escovar et al., 1996), *diap1-lacz* (BL 12093), *UAS-Yki^{S168A}* (BL 28818), *ex-lacz* (BL 44248), *Mi-2 RNAi* (BL 51774), *Mi-2 RNAi-2* (BL33419), *Sin3A RNAi* (BL 32368).

2.2. Mosaic clone induction

Heat shock Flp-out system used to induce clones with ectopic expression of protein or RNAi in our studies is based on UAS/GAL4, FLP/FRT cassette, and *in vivo* RNAi methods (Brand and Perrimon, 1993; Ni et al., 2008; Pignoni and Zipursky, 1997). To generate the clones, 24–48 h after egg deposition (AED) *Drosophila* larvae with *hs-FLP Act>CD2-Gal4 UAS-GFP* and UAS driven protein coding cDNA and/or RNAi were heat shocked at 34 °C for 15 min to 1 h, depending on the clone sizes of each genotype. The imaginal discs were dissected from larvae at 48–72 h after the heat shock for fixation and staining. Except for the heat shock clone induction, all flies for the experiments were kept at 25 °C.

2.3. Immunostaining

Immunostaining and imaging were done with the protocols as described in our previous studies (Gordon et al., 2013; Zhang et al., 2014). Primary antibodies used in this study include: mouse anti-β-Galactosidase (1:100, DSHB), mouse anti-DLG (1:100, DSHB), rabbit anti-activated Caspase-3 (C3, 1:500, Cell Signaling), rabbit anti-Yki antibody (1:400) (Oh and Irvine, 2008). Secondary antibodies are from Jackson ImmunoResearch (1:200 to 1:400).

2.4. Drosophila genotypes used in each figures

2.4.1 Figure 1

A, B, E, G: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Rpd3 RNAi/+*

D, F: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+;*

C: *dpp-Gal4, UAS-GFP/+; UAS-Rpd3 RNAi-2/+*

2.4.2 Figure 2

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz/+*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz UAS-Rpd3 RNAi/+*

E, F: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz UAS-Rpd3 RNAi/UAS-Puc*

G, H: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Rpd3 RNAi/UAS-Puc*

2.4.3 Figure 3

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz/+*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz UAS-Rpd3 RNAi/+*

E, F: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz UAS-Rpd3 RNAi/UAS-Yki^{S168A}*

G, H: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Rpd3 RNAi/UAS-Yki^{S168A}*

2.4.4 Figure 4

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz UAS-Rpd3 RNAi/UAS-Puc*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz UAS-Rpd3 RNAi/UAS-Yki^{S168A}*

E, F: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz UAS-Rpd3 RNAi/UAS-Puc UAS-Yki^{S168A}*

2.4.5 Figure 5

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz/UAS-Mi-2 RNAi*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz/UAS-Mi-2 RNAi*

E, G: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Mi-2 RNAi/+*

F, H: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Mi-2 RNAi/UAS-Puc*

2.4.6 Figure 6

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz/UAS-Sin3A RNAi*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz/UAS-Sin3A RNAi*

2.4.7 Figure S1

yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz/UAS-Rpd3 RNAi-2

2.4.8 Figure S2

yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Rpd3 RNAi puc-lacz/UAS-P35

2.4.9 Figure S3

yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz/UAS-Rpd3 RNAi-2

2.4.10 Figure S4

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; ex-lacz/+; UAS-Rpd3 RNAi/+*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; UAS-Rpd3 RNAi/+*

2.4.11 Figure S5

A, B: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; puc-lacz/UAS-Mi-2 RNAi-2*

C, D: *yw, hsFLP, Act>CD2>Gal4, UAS-CD8GFP/+; diap1-lacz/UAS-Mi-2 RNAi-2*

3. Results

3.1. Inactivation of Rpd3 induces apoptosis

Previous study showed that *Rpd3* mutant clones were tiny in *Drosophila* imaginal discs (Zhu et al., 2008), which makes it challenging to further analyze the underlying molecular mechanisms. To overcome this problem, we used the *hs-FLP/FRT* cassette and the *UAS/Gal4* based "heat shock flp-out" system to deplete *Rpd3* by RNAi. With this system, the *Rpd3-RNAi* clones in imaginal discs were labeled with the co-expressed GFP and the levels and the consequences of *Rpd3* knockdown were controlled by the amount of time after heat shock. As expected, very few surviving *Rpd3-RNAi* clones were observed in either eye or wing discs at 72 h after clone induction. The level of activated Caspase3 (C3) is correlated with cell apoptosis in *Drosophila* imaginal discs. Staining with C3 antibody showed high C3 level in the *Rpd3-RNAi* clones by comparing with surrounding *WT* cells (Fig. 1A–B). Similarly, constitutive expression of another *Rpd3 RNAi* construct, *Rpd3 RNAi-2*, that targets a distinct part of *Rpd3* mRNA, also induced strong C3 level (Fig.