RISC hitches onto endosome trafficking

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The RNA-induced silencing complex (RISC) downregulates expression of the genes targeted by RNA-silencing pathways. But formation and turnover of the RISC complex itself is tightly regulated and requires endosomal membranes.

Over the past few years, we have seen remarkable progress in our understanding of how small non-coding RNAs of 20–30 nucleotides, including small interfering RNAs (siRNAs) and microRNAs (miRNAs)[1], guide the downregulation of gene expression. These small regulatory RNAs associate with Argonaute (AGO) proteins to form RISCs that direct degradation or translational repression of target mRNAs. AGO proteins colocalize with P-body components, where enzymes involved in mRNA decapping (Dcp1 and Dcp2) and decay are concentrated, suggesting a physical interaction between components of RISCs and P-bodies[2,3]. Targeting mRNAs away from the translational machinery into P-bodies could be an important mechanism in miRNA- and siRNA-mediated silencing. However, where in the cell RISC formation and/or turnover occurs remains unclear. In two complementary papers on pages 1143 and 1150 of this issue, Gibbings *et al.*[4] and Lee *et al.*[5] show that RISC formation and turnover depends on the endosomal pathway. These results are of interest not just for the insights they provide into the processes of RISC formation and turnover, but also because they provide a link between RNA silencing and membrane trafficking[6].

Many plasma-membrane proteins are internalized by endocytosis and are sorted to their final destination by the endosomal pathway[6]. Endosome maturation involves a gradual accumulation of internal vesicles (Fig. 1a). Late endosomes will have accumulated several hundred luminal vesicles that arise from internal budding of the endosomal membrane. These endosomes are often referred to as multivesicular bodies (MVBs). They can fuse with lysosomes, resulting in degradation of their cargo. MVBs can also fuse with the plasma membrane, in an exocytic manner, to release internal vesicles (exosomes) into the extracellular milieu[7] (these should not be confused with the ribonuclease complex that is also known as an exosome). The formation of inwardly budding vesicles in endosomes and the sorting of selected cargo into these vesicles requires the sequential assembly of four ESCRT (endosomal sorting complex required for transport) complexes on the endosomal membrane; these complexes are well conserved across the eukaryotic lineage. Ubiquitylated cargoes engage the ubiquitin-binding domain of ESCRT components for sorting into these vesicles.

Exosomes were recently shown to contain mRNAs and miRNAs[8,9] and can transfer these nucleic acids into a recipient cell. Consistent with these findings, Gibbings *et al.*[4] purified exosome-like vesicles from cultured monocytes and found that they contained a large number of miRNAs. They also contained low levels of AGO2 as well as dramatically high levels of the P-body component GW182, which is required for miRNA function through its binding to AGO proteins[2]. Knockdown of GW182 disrupts P-bodies and significantly impairs the ability of both miRNAs and siRNAs to repress target gene expression[2]. However, the components of the P-body decapping complex, DCP1A and GE-1, were barely detected in purified exosome-like material.

Subsequent membrane fractionation analysis showed that GW182 and AGO2 were enriched in the endosomal/MVB fraction, suggesting that crucial components of the RISC congregate in endosomes and MVBs[4]. These results are consistent with the initial identification of AGO2 as a membrane-associated protein[10]. Gibbings *et al.* demonstrate that at least two pools of AGO2-associated RNA-silencing components exist. One pool, defined as GW bodies, is GW182-rich, DCP1A-poor and is associated with MVB components. The second pool does not contain membrane-associated proteins, is DCP1A-rich and GW182-poor and is therefore defined as P-bodies. These findings suggest that the AGO2 and GW182 foci that colocalize with MVBs constitute subcellular structures distinct from P-bodies (Fig. 1a). The findings also contradict previous results supporting a strong association of GW182 with P-bodies[2,3]. Gibbings *et al.* propose that GW182 aggregates often, albeit not always, correspond to endosomes/MVBs. These results also raise the possibility that MVBs constitute sites of RISC assembly and/or function. Consistent with this proposal, miRNA-repressible mRNAs accumulated in fractions corresponding to MVBs. However, miRNA-repressible mRNAs are markedly under-represented in exosome-like vesicles. Thus, whereas miRNA-repressible mRNAs are enriched in GW182- and AGO2-associated membranous fractions, they seem selectively excluded from exosome-like vesicles. These results suggest that a pool of GW182 selectively dissociates from membrane-bound, miRISC–mRNA complexes to be sorted into MVBs and is subsequently secreted or degraded through the exosome–lysosome pathway (Fig. 1b).

Gibbings *et al.* found that blocking MVB formation by knockdown of the ESCRT component genes *hrs* and *alix* results in an increase in cellular GW182, but not DCP1A, levels. Furthermore, knockdown of *hrs* and *alix* inhibited miRNA activity, as monitored by let-7a and its target reporter, and also affected let-7a accumulation. Thus, blocking the formation of MVBs generally compromises miRNA functions, probably by interfering with the sorting of GW182 into MVBs. Gibbings *et al.* hypothesize that ESCRT-dependent removal of GW182 molecules into MVBs might constitute a limiting step in miRNA loading onto AGO, or might allow continuous assembly and disassembly of the membrane-associated RISC, required for its maturation or function (Fig. 1).

Independently, Lee *et al.* carried out a screen in *Drosophila* to identify mutations that enhance siRNA- and miRNA-mediated gene silencing. One of the mutated genes is orthologous to the gene responsible for a rare autosomal recessive disease — Hermansky-Pudlak Syndrome 4 (*HPS4*)[11]. Loss of HPS4 can also enhance RNAi in mammalian cells,

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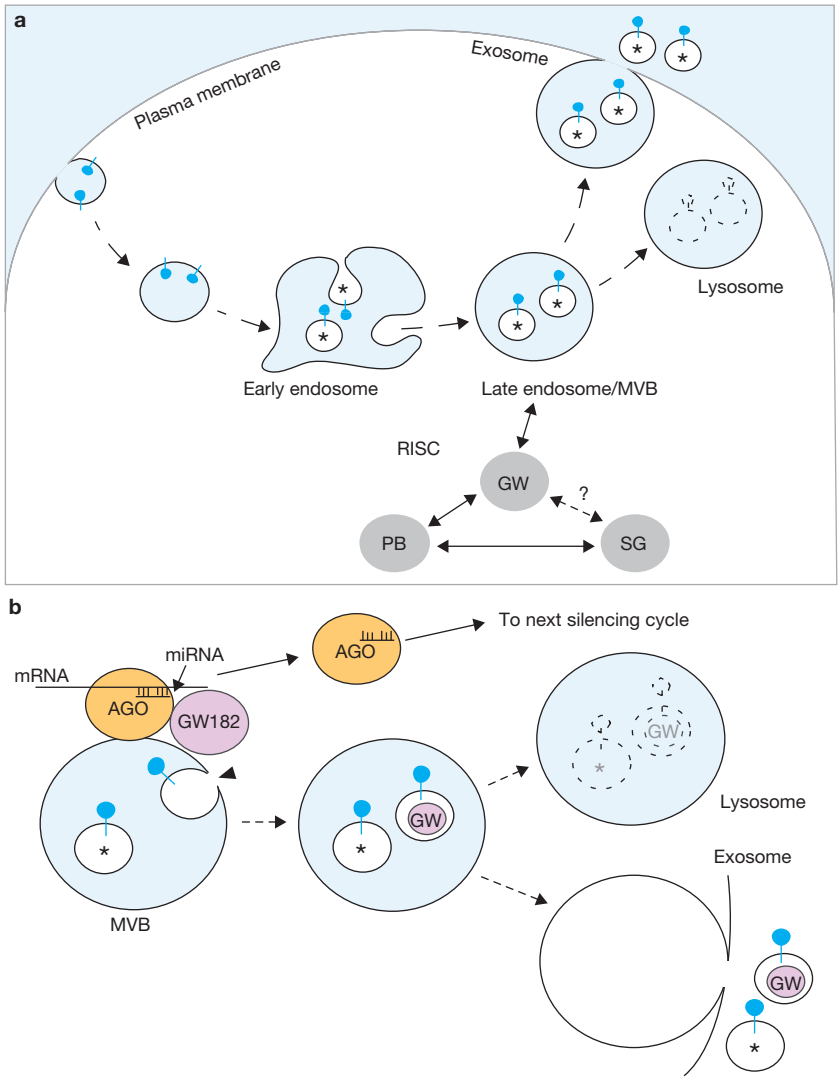

Figure 1 RISC assembly and turnover occurs at endosomes. (**a**) Membrane proteins (blue) are brought inside the cell by incorporation into endocytic vesicles that fuse with an early endosome. Multivesicular bodies (MVBs) are late endosomal compartments, between early endosomes and lysosomes. MVBs can also release internal vesicles, called exosomes, into their extracellular space. RISC formation and/or turnover appears to occur on MVBs. GW182 aggregates (GW) are often colocalized with MVBs and define subcellular structures distinct from P-bodies (PB), the cytoplasmic aggregates that contain translationally repressed ribonucleoprotein particles and are associated with the translation repression and mRNA decay machinery. SG represents stress granules. (**b**) Blocking the fusion of MVBs with lysosomes or the plasma membrane stimulates RISC activity. In contrast, blocking the ESCRT-dependent formation of MVBs from early endosomes inhibits RISC activity. Thus it is hypothesized that ESCRT-dependent removal of GW182 into MVBs might be a limiting step in miRNA loading onto AGO, or might allow the continuous assembly and disassembly of membrane-associated RISCs, required for their maturation or function. Asterisks represent cargoes in exosomes.

indicating that HPS4 attenuates RNAi across the animal kingdom. The *HPS4* gene is known to be required for the fusion of MVBs with lysosomes[11]. Interestingly, Lee *et al.* found that mutations in the ESCRT genes *hrs* and *vps25* also impair miRNA-mediated silencing. In contrast to the effect of mutations in the *HPS4* gene, the authors showed that blocking the formation of MVBs from early endosomes could inhibit RISC activity (Fig. 1b).

In *Drosophila HPS4*-mutant cells, AGO1, a key component of miRNA–RISC (miRISC)[1], was dispersed throughout the cytoplasm. In contrast, mutations in the ESCRT genes that block sorting of early endosomes into MVBs and thereby generate enlarged early endosomes, resulted in the concentration of AGO1 around enlarged early endosomes. These findings suggest that blocking MVB formation concentrates miRISCs around early endosomes whereas blocking MVB turnover disperses miRISCs from lysosomes to the cytosol. Surprisingly, the levels of miRNA associated with AGO1 were greater when AGO1 was immunopurified from *Drosophila HPS4*-mutant cells, indicating that miRISC loading is enhanced when MVB turnover is impaired.

Maturation of early endosomes into MVBs depends upon the recognition of ubiquitylated proteins in early endosomes by ESCRT proteins[6]. Lee *et al.* demonstrate that mutations in a *Drosophila* ubiquitin ligase impair silencing mediated both by siRNAs and miRNAs. A dominant-negative form of ubiquitin also inhibits loading of AGO2 with exogenously labelled siRNA. Together, these findings suggest that protein ubiquitylation stimulates RISC activity through enhanced small RNA loading onto RISCs, although effects through additional pathways cannot be excluded.

Gibbings *et al.* and Lee *et al.* thus provide evidence that supports a model in which active miRISCs associated with target mRNAs are recruited into GW-bodies that are physically associated with MVBs. Alternatively, but not mutually exclusively, MVBs might regulate silencing by increasing the ability of miRISCs to load miRNAs, perhaps by promoting turnover of miRISCs (Fig. 1b). Reduced secretion or degradation would cause accumulation of GW182; however, this would concomitantly compromise the proposed assembly or turnover of RISCs. GW182 directly interacts with AGO proteins. Thus, the GW182 association with AGO may function as a temporal lock that could compete with Dicer for loading empty AGO with miRNA, or for reloading AGO with new miRNA after an initial round of miRNA-dependent repression. MVBs may be the sites where miRNA biogenesis and RISC-assembly pathways intersect, with continuous removal of GW182 being required for the efficient passage of miRNA–miRNA antisense strand duplex from DICER to AGO. Consistent with this idea, AGO1 associates with Dicer-1 and GW182 in a mutually exclusive manner in *Drosophila*[12]. How AGO is dissociated from GW182 remains to be elucidated.

These new findings should help define the molecular pathways involved in RNA silencing. They also underline the possibility that small RNAs could be secreted from cells through exosome pathways, for regulatory purposes. Finally, the ESCRT machinery is used by enveloped retroviruses for outward virus budding at the plasma membrane, a process topologically

identical to inward budding away from the cytoplasm to form MVBs[6]. These new results raise the possibility that some retroviruses such as HIV-1 may contain GW182, miRNAs and some AGO proteins within their virions, perhaps allowing them to use these RNA-silencing components in the arms race with host cells.

1. Siomi, H. & Siomi, M. C. *Nature* **457,** 396–404 (2009).
2. Eulalio, A. *et al. Nature Rev. Mol. Cell Biol.* **8,** 9–22 (2007).
3. Parker, R. & Sheth, U. *Mol. Cell* **25,** 635–646 (2007).
4. Gibbings, D. J., Ciaudo, C., Erhardt, M, & Voinnet, O. *Nature Cell Biol.* **11,** 1143–1149 (2009).
5. Lee, Y. S. *et al. Nature Cell Biol.* **11,** 1150–1156 (2009).
6. Raiborg, C. & Stenmark, H. *Nature* **458,** 445–452 (2009).
7. Stoorvogel, W. *et al. Traffic* **3,** 321–330 (2002).
8. Valadi, H. *et al. Nature Cell Biol.* **9,** 654–659 (2007).
9. Skog J. *et al. Nature Cell Biol.* **10,** 1470–1476 (2008).
10. Cikaluk, D. E. *et al. Mol. Biol. Cell.* **10,** 3357–3372 (1999).
11. Li, W. *et al. Bioessays* **26,** 616–628 (2004).
12. Miyoshi, K. *et al. RNA* **15,** 1282–1291 (2009).

Steroid hormone pulsing drives cyclic gene expression

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Transcriptional cycling of activated glucocorticoid receptor (GR) and ultradian glucocorticoid secretion are well established processes. Ultradian hormone release is now shown to result in pulsatile gene transcription through dynamic exchange of GR with the target-gene promoter and GR cycling through the chaperone machinery.

Endogenous glucocorticoid secretion by adrenal glands is controlled by the hypothalamic-pituitary axis, resulting in a circadian (every 24 hours) and an ultradian (every hour) pattern of hormone release into the blood stream[1]. On page 1093 of this issue, Stavreva *et al.* show how nuclear GR translates the natural, ultradian glucocorticoid pulses into a molecular response of transcriptional pulses, an effect that is not observed with synthetic glucocorticoids[2].

Endogenous, active glucocorticoids, cortisol in humans and corticosterone in rodents, are key pleiotropic hormones regulating metabolism, stress response and the immune system in adults[3]. Synthetic glucocorticoids are widely used to treat chronic inflammatory diseases[3,4]. GR, a steroid hormone nuclear receptor specific for glucocorticoids, is expressed in virtually all cells, where it is required for glucocorticoid-dependent gene expression. GR undergoes rapid cycling on and off the promoters of its targets in the presence of natural glucocorticoids[5,6]. In addition, the highly dynamic association of GR and cortisol allows GR to sense rapid changes in cortisol concentration, which fluctuates, *in vivo,* in a circadian and an ultradian manner[1]. Stavreva *et al.* use an engineered mouse cell line that expresses fluorescent GR and contains an amplified array of GR-responsive promoter structures, including GR response elements, to monitor GR association with the promoters in live cells.

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The authors show that successive exposure to hormone drives dynamic cycles of association and dissociation of GR with the promoter array. These cycles are mirrored by pulses of transcriptional activity, which directly correlate with the flux in hormone levels. In contrast, synthetic glucocorticoids lead to GR association with the promoter array, but the receptor fails to dissociate after hormone washout (withdrawal), leading to sustained target gene expression. Moreover, the authors found that *in vivo*, when adrenalectomised rats were supplied with a natural glucocorticoid in an ultradian manner, both GR association with target promoters and gene expression responded dynamically to glucocorticoid levels, which was similar to their observations in cell cultures. These findings highlight fundamental mechanisms of transcriptional regulation in single cells and demonstrate a rapid and dynamic GR-mediated transcriptional response to natural hormone pulses *in vivo*.

Oscillation in the transcriptional activity of various transcription factors is well established and has been proposed to be a common feature of gene regulation[7]. Nuclear receptors, in particular, have been the focus of detailed studies. In a simple model, nuclear receptor-dependent transcriptional cycling depends on hormone availability, association and dissociation of the nuclear receptor with and from its cognate response element and removal of the nuclear receptor and its cofactors through proteasomal degradation. These steps first generate transcriptional competence followed by productive transcription and finally limit transcription through promoter clearance[8].

In such a scheme, the hormone itself is not a cycling component. The following cycling events have been described for GR: a slow, hormone-dependent nucleo-cytoplasmic shuttling of GR; continuous dissociation and re-association of GR and hormone in the nucleus (occurring in minutes) and rapid exchange of GR on the GR response element (occurring in seconds)[5,9]. Chaperones have a role in each of these three cycles (Fig. 1). Ultradian rhythm of hormone stimulation adds yet another time scale, in the order of an hour, and must be integrated into the model for GR-driven transcription. When independently analysed, all of the above-mentioned oscillations contribute to GR-mediated transcriptional activity. However, the complexity generated by these additive layers of dynamic patterns makes it difficult to predict transcriptional activity at a given time point.

So, what is the biological relevance of GR-driven gene pulsing? One proposal is that nuclear receptor-driven cyclic transcription may allow target gene transcription to rapidly adjust to hormone fluctuations. This recent study indeed demonstrates that ultradian oscillations of glucocorticoid are temporally followed by transcriptional oscillations. But why the need for such reactivity? Ultradian and circadian rhythms of glucocorticoid fluctuation are thought to allow synchronization and coordination of diurnal and nocturnal events. In addition, glucocorticoid response can be triggered by stress, at any time, to facilitate adaptation and contribute to restoring homeostasis.