Processing of primary microRNAs by the Microprocessor complex

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Mature microRNAs (miRNAs) are generated via a two-step processing pathway to yield ~22-nucleotide small RNAs that regulate gene expression at the post-transcriptional level[1]. Initial cleavage is catalysed by Drosha, a nuclease of the RNase III family, which acts on primary miRNA transcripts (pri-miRNAs) in the nucleus[2]. Here we show that Drosha exists in a multi-protein complex, the Microprocessor, and begin the process of deconstructing that complex into its constituent components. Along with Drosha, the Microprocessor also contains Pasha (partner of Drosha), a double-stranded RNA binding protein. Suppression of Pasha expression in *Drosophila* cells or *Caenorhabditis elegans* interferes with pri-miRNA processing, leading to an accumulation of pri-miRNAs and a reduction in mature miRNAs. Finally, depletion or mutation of *pash-1* in *C. elegans* causes de-repression of a *let-7* reporter and the appearance of phenotypic defects overlapping those observed upon examination of worms with lesions in Dicer (*dcr-1*) or Drosha (*drsh-1*). Considered together, these results indicate a role for Pasha in miRNA maturation and miRNA-mediated gene regulation.

miRNAs are a class of small, non-coding RNAs that enter the RNA interference (RNAi) pathway to regulate the expression of protein-encoding genes at the post-transcriptional level[3]. miRNA production begins with the synthesis of pri-miRNAs, ranging in size from several hundred nucleotides (nt) to several kilobases. These are recognized and cleaved into precursor miRNAs (pre-miRNAs) in the nucleus by an RNase III family nuclease, Drosha[2]; the pre-miRNAs are short, hairpin RNAs of approximately 70 nt, bearing the 2-nucleotide 3′ overhang that is a signature of RNase III-mediated cleavage. Pre-miRNAs are exported to the cytoplasm by a RanGTP/exportin 5-dependent mechanism[4–6], with their characteristic overhang contributing to their entry into this export pathway[5]. Once in the cytoplasm, pre-miRNAs are recognized and processed into their mature, ~22-nt form by Dicer[7–9], with the 3′ overhang again playing a role in specifying cleavage[10–12]. Mature miRNAs enter RISC (RNA-induced silencing complex) in an asymmetric fashion such that for most miRNAs, only one strand is enabled to recognize and repress the expression of target genes[13]. The outcome of this recognition is either endonucleolytic cleavage of the targeted messenger RNA[14,15] or interference with protein synthesis by a mechanism that remains unclear[16–18]. With the ultimate goal of addressing the mechanisms by which pri-miRNAs are tagged for entry into the RNAi pathway and processed at specific sites, we have undertaken a biochemical characterization of Drosha and its associated factors.

Previously, Kim and colleagues showed[2] that epitope-tagged, human Drosha protein was capable of releasing pre-miRNAs from pri-miRNAs *in vitro* and contributed to miRNA maturation *in vivo*. Processing was dependent upon the presence of a double-stranded region around the cleavage position. We asked whether a pri-miRNA processing activity also existed in *Drosophila* cells. Although they are absent from *Schizosaccharomyces pombe* and *Arabidopsis*, homologues of mammalian Drosha are present in *Drosophila* and *C. elegans*[19]. Indeed, S2 cell extracts contain an activity that can recognize a pri-miRNA, in this case pri-bantam, and cleave this into discrete products that can be identified as pre-bantam and the regions of the pri-miRNA that flank that mature sequence; pre-bantam and the 5′ and 3′ flanks are 60, 104 and 112 nt, respectively (Fig. 1a; see Supplementary Fig. S1 for sequences). Immunoprecipitates obtained using an affinity-purified Drosha antibody were capable of generating pre-bantam from pri-bantam (Fig. 1b). Examination of the supernatants showed that a substantial fraction of pri-miRNA processing activity was depleted from the extract by immunoprecipitation (data not shown). Additional pri-miRNAs from both human and *Drosophila* were similarly processed by extracts and immunoprecipitates (data not shown). Considered together, these results implicate Drosha and/or

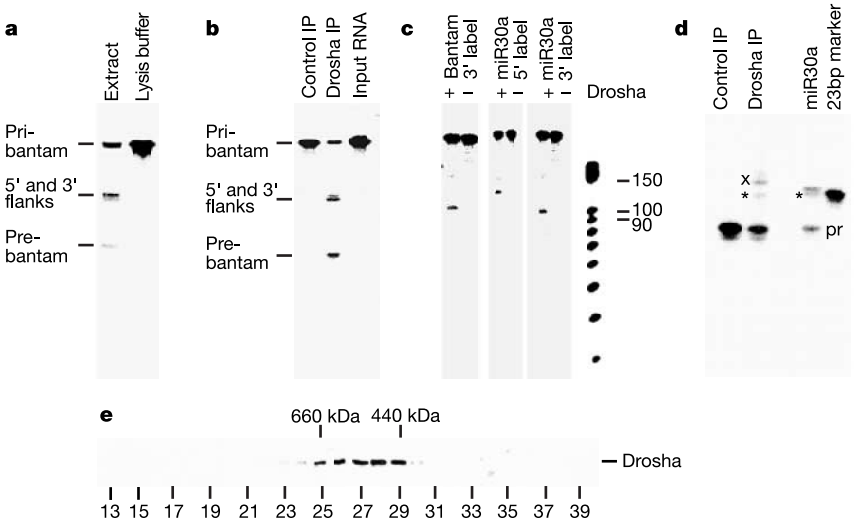

Figure 1 Pri-miRNA processing is conserved in *Drosophila*. **a**, Pri-miRNA processing activity was assessed in *Drosophila* S2 cell extracts using uniformly labelled pri-bantam miRNA. Positions of the processed pre-bantam and the two flanking RNAs derived from excision of pre-bantam are indicated. **b**, Immunoprecipitation experiments with S2 extracts test whether processing activity associates with Drosha. **c**, To assess the accuracy of pri-miRNA processing, cleavage sites were mapped using end-labelled substrates. Pre-bantam or pre-miR30a was 3′ labelled by ligation to ^{32}P-pCp, and pri-miR30a was 5′ labelled by capping with ^{32}P-GTP. Sizes of cleavage products were as predicted (see Supplementary Fig. S1). RNA size markers, indicated in bases, are shown at the right. **d**, The 5′ end of processed pre-miR30a was mapped by primer extension. Control extension of a synthetic pre-miR30a RNA is shown for reference along with a 23 bp 5′-end-labelled DNA marker. **e**, Western blots with anti-Drosha antibody across fractions of a Superose 6 column show that Drosha from S2 cells exists in a ~500 kDa complex. Fraction numbers are given below the panel.

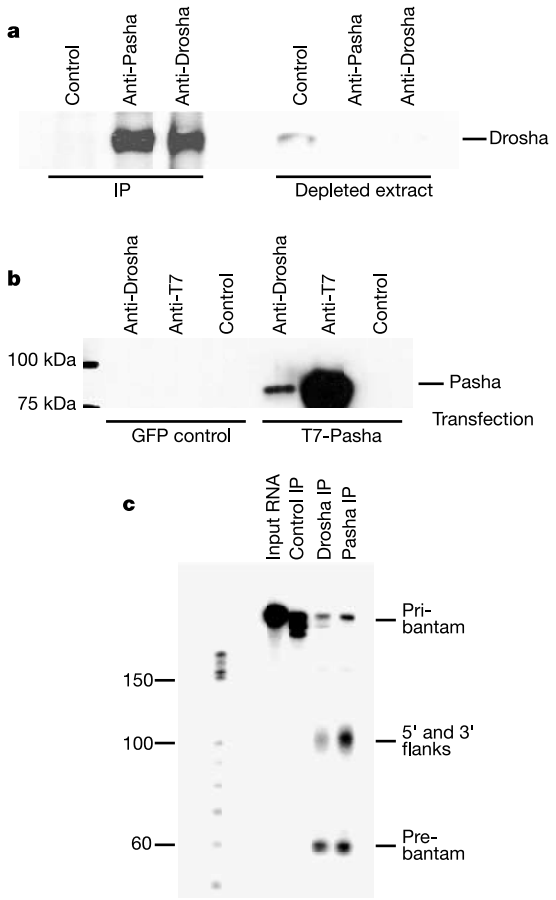

Figure 2 Pasha, a nuclear dsRNA binding domain (dsRBD) protein, associates with Drosha. **a**, Immunoprecipitates from S2 cell extracts with antibodies directed against endogenous Drosha or Pasha proteins were examined by western blotting with anti-Drosha antibody. To assess the degree of co-association, supernatants were also probed. The immunoprecipitates were derived from extract equivalent to ~40 × of what was loaded in the supernatant lanes. **b**, Immunoprecipitations were prepared from S2 cells that were transfected with either a GFP expression construct or a construct expressing T7 epitope-tagged Pasha. **c**, Immunoprecipitates were incubated with uniformly labelled pri-bantam and tested for processing activity.

its associated factors as the major source of pri-miRNA processing activity in *Drosophila* extracts.

The accuracy of pri-miRNA processing by our immunoaffinity-purified Drosha was assessed in two ways. First, we examined the processing of two pri-miRNA substrates that were labelled at their 5′ or 3′ termini (Fig. 1c). Cleavage of pri-bantam or pri-miR30a yielded the expected end fragments. The end of pre-miR30a has previously been mapped by primer extension of *in vivo* and *in vitro* processed transcripts[1]. Upon analysis of pri-miR30a, processed using immunoaffinity-purified *Drosophila* Drosha, we find two prominent extension products. The smaller (denoted with an asterisk in Fig. 1d) is consistent with the expected pre-miR30a product, based both on a labelled DNA marker and on primer extension of a synthetic version of the expected pre-miR30a product. A larger product (denoted by 'X' in Fig. 1d), differing from the expected product by 2–3 nt, is of unknown origin. However, it should be noted that many nucleases, including Dicer, are known to make multiple cleavages within their substrates. In contrast, Drosha does not operate on fully duplexed substrates[20] (Supplementary Fig. S2).

To investigate how Drosha selects its substrates and how the enzyme determines its specific cleavage sites, we sought to characterize native complexes containing the enzyme. Biochemical fractionation indicates that Drosha is present within an ~500 kDa complex, which presumably contains additional protein components (Fig. 1e). Given its role in microRNA metabolism, we have dubbed this complex the Microprocessor.

Recent, genome-wide two-hybrid analysis of *Drosophila* has generated a substantial list of candidate protein–protein interactions[21]. In examining the list, we noted a potential interaction between Drosha and a double-stranded RNA (dsRNA) binding protein, originally dubbed CG1800 (candidate gene 1800). This protein consists of two domains, a WW domain near the amino terminus and a dsRNA binding domain near the carboxyl terminus. Notably, WW domains interact with proline-rich domains, such as the one present near the N terminus of Drosha. Along with Drosha, CG1800 is conserved in *C. elegans* and mammals, where it was called DGCR8[22]. Like Drosha, CG1800 homologues are absent from the *Arabidopsis* and *S. pombe* genomes. Results from the published two-hybrid studies, as well as evidence of physical and genetic interaction presented below, caused us to designate CG1800 as Pasha (partner of Drosha).

Consistent with the possibility that they might both be present in a single complex, Drosha and Pasha are predominantly nuclear

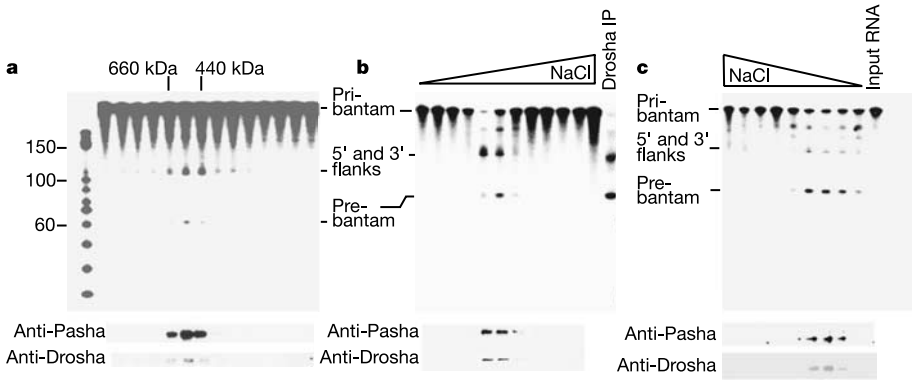

Figure 3 Drosha and Pasha co-exist in a Microprocessor complex. **a**, Combined nuclear and cytoplasmic extracts from S2 cells were loaded onto a Superose 6 column, and fractions were subjected to western blotting with Drosha or Pasha antibodies (lower panel). The Drosha antibody was used to recover Drosha complexes from each fraction and these were tested for their ability to process pri-bantam (upper panel). Positions of pre-bantam and the 5′ and 3′ flanks are indicated. **b**, Extracts prepared as in **a** were run on a Q sepharose FF column. Drosha and Pasha fractionation profiles were determined by western blotting (lower panels). Pri-miRNA processing activity on pri-bantam was examined using Drosha immunoprecipitates from each fraction. Direct analysis of processing activity in each fraction showed a similar processing activity profile, albeit with weaker activity than exhibited by the immunoprecipitates. **c**, Drosha peak fractions from a Q-sepharose column were pooled and loaded onto a phenyl HP column. Fractionation of Drosha and Pasha and processing activity were followed as in **b**.

proteins (Supplementary Fig. S3; data not shown). Furthermore, Pasha antiserum co-immunoprecipitates Drosha, essentially depleting it from whole cell extracts (Fig. 2a). This interaction was not disrupted by treatment of extracts or immunoprecipitates with RNase (data not shown). Analysis of the reciprocal immunoprecipitate required the use of a T7-tagged Pasha, since the antibody heavy chain interferes with detection of this protein in western blots with the rabbit anti-Pasha antibody. In T7-Pasha-expressing cells, a Drosha antibody co-immunoprecipitates tagged Pasha (Fig. 2b). Drosha–Pasha complexes are functional since both Drosha and Pasha immunoprecipitates are able to process pri-miRNA into pre-miRNA *in vitro* with similar efficiencies (Fig. 2c). To test whether Drosha and Pasha coexist in the 500 kDa Microprocessor, we examined chromatographic profiles from S2 cell extracts. Both

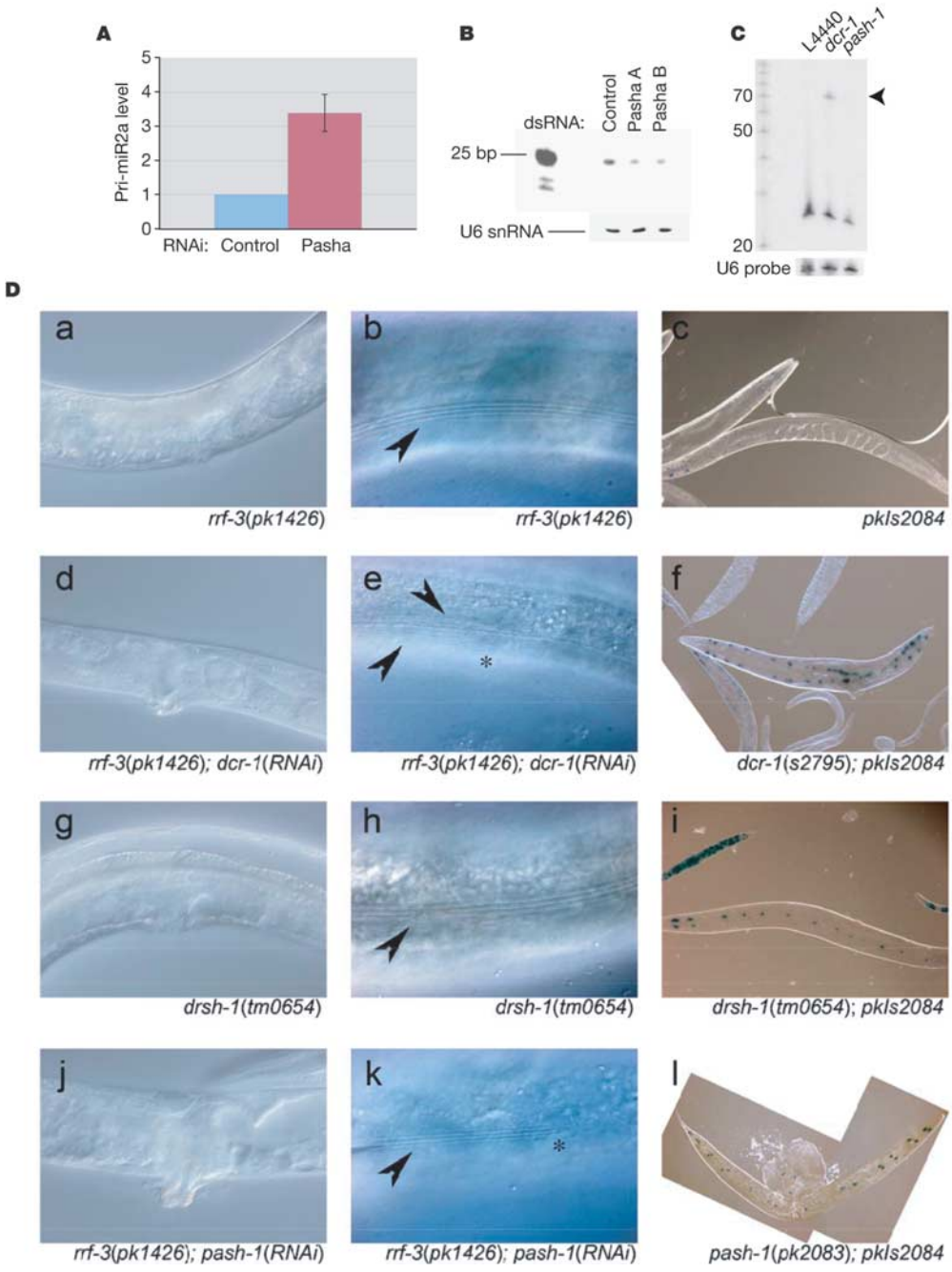

Figure 4 Depletion of Pasha reduces pri-miRNA processing. **A**, Pri-miR2a levels were analysed following transfection of *Drosophila* S2 cells with two different dsRNAs homologous to *Pasha* by semiquantitative RT–PCR. Error bar indicates standard deviation from the mean. **B**, Total RNA extracted from S2 cells transfected with either a control dsRNA (luc) or either of two Pasha dsRNAs was examined for the level of mature miR2a by northern blotting. U6 snRNA was used as a control to ensure equal loading. **C**, *C. elegans* (*rrf-3* mutant) were treated with Pasha (*pash-1*), Dicer (*dcr-1*) or control dsRNAs (L4440), and total RNA was prepared. Levels of pre- and mature *let-7* were assessed by northern blotting. Sizes of selected RNA markers are indicated in bases at the left. **D**, Phenotypic analysis of Pasha (*pash-1*) and Drosha (*drsh-1*) in *C. elegans* was performed using RNAi and genetic mutants. RNAi against *pash-1* leads to vulva (100%) and alae (10/17) defects, similar to those published for *dcr-1* mutants[8,9] (**a** and **b**, **d** and **e**, **g** and **h**, and **j** and **k**). The observed defects include protrusion and bursting of the vulva, and gaps in, or absence of the alae (asterisks). Occasionally duplicated alae are detected, as shown in **e**. The *drsh-1(tm0654)* allele does not result in obvious defects in these structures (alae indicated by arrow heads); however this mutant may be partially rescued by maternal contribution of Drosha. **i** and **l**, both *drsh-1(tm0654)* (10 out of 10) and *pash-1(pk2083)* (14 out of 14) result in defects in the *let-7*-mediated silencing of a previously described transgene (*pkIs2084*)[24]. This defect is similar to that observed in a *dcr-1*(*s2795*) mutant (**f**: 15 out of 15). In wild-type adults, the *lacZ* expression is silenced (**c**: 0 out of 57 showing *lacZ* expression).

proteins along with pri-miRNA processing activity peaked in an ~500 kDa fraction (Fig. 3a). Similar co-fractionation was also seen if complexes were serially chromatographed on Q sepharose FF and phenyl HP columns (Fig. 3b, c) or on Q sepharose FF and mono S (data not shown).

To examine the functional connection between Pasha and miRNA metabolism, we asked whether suppression or mutation of Pasha had any affect on pri-miRNA processing or miRNA function. In *Drosophila* S2 cells, Pasha dsRNAs caused a modest suppression of *Pasha* mRNA (Supplemental Fig. S4). This resulted in both an accumulation of pri-miR2a and a reduction in levels of mature miR2a (Fig. 4a, b). Similar effects on pri-miR30a were seen upon transfection of mammalian cells with two different human *Pasha* siRNAs (Supplementary Fig. S5). Similarly, treatment of *C. elegans* with *pash-1* dsRNA, by feeding of RNAi-hypersensitive *rrf-3* mutant worms, resulted in an accumulation of pri-*let-7* with a decrease in the mature species (Fig. 4c; Supplementary Fig. S6). Notably, similar effects were seen upon suppression of Dicer (*dcr-1*), although in this case the pre-miRNA also accumulated. A comparison of these two results suggests that Pasha acts in pri-miRNA metabolism upstream of pre-miRNA production, consistent with its placement by biochemistry as a component of the Microprocessor. Precisely why pri-miRNAs also accumulate in worms treated with Dicer dsRNA but not in worms treated with control dsRNAs is at present unclear.

For studies of the biological function of Drosha and Pasha *in vivo*, we again turned to *C. elegans*. A deletion allele of Drosha, *drsh-1(tm0654)* causes a sterile phenotype, with no other visible defects. Specifically, we looked at the presence of alae and the structure of the vulva (Fig. 4D, g and h), as these structures are often affected by lesions in miRNA pathway genes like *dcr-1* (Fig. 4D, d and e)[8,9]. Most probably, such defects are not observed in *drsh-1(tm0654)* animals because of a strong maternal rescue. Defects in the alae and vulva structures are easily detected in *pash-1* RNAi knock-down animals and to a lesser degree in a *pash-1(pk2083)* nonsense mutant (Fig. 4D, j and k; data not shown). Typical defects include protrusion or bursting of the vulva, and gaps or absence of the alae. In addition, we used a more sensitive and specific assay to directly monitor the activity of the *let-7* miRNA. The assay is based on a *lacZ* reporter that is silenced by *let-7* through sequences in the 3′ UTR[23]. This results in detectable *lacZ* staining in all larval stages because *let-7* is not yet expressed in these stages, but an absence of *lacZ* in the *let-7* expressing adult (Fig. 4D, c). As a control, we used *dcr-1* mutant animals. Indeed, in this mutant background *lacZ* is reactivated in the adult (Fig. 4D, f)[24]. We then asked whether the adult-specific silencing of this reporter requires *drsh-1* and/or *pash-1*. Both *pash-1(pk2083)* and *drsh-1(tm0654)* lead to re-expression of the reporter in the adult stage (Fig. 4D, l and i), indicating that *in vivo*, both Drosha and Pasha proteins are required for the function or synthesis of the *let-7* miRNA.

Considered together, our results indicate that Pasha and Drosha are components of a multiprotein machine, the Microprocessor, which converts pri-miRNAs into pre-miRNAs. Although the experiments presented here do not permit us to assign a definitive function to Pasha within the Microprocessor, it is reasonable to speculate that Pasha might have one of several roles. For example, it could help in identifying primary miRNA transcripts, facilitating delivery of these to Drosha for cleavage. Indeed, Pasha immunoprecipitates contain pri-miRNAs (Supplementary Fig. S7). Alternatively, Pasha could help to orient the pri-miRNA in the Microprocessor, contributing to the specific positioning of the Drosha cleavage site. A parallel study shows an association between DGCR8 and human Drosha, and presents biochemical and genetic evidence that these proteins cooperate to determine the specificity of Drosha cleavage[25]. On the basis of the results presented here, Pasha joins RDE-4, Hyl-1 and R2D2[26–28] to form a growing list of dsRNA-binding proteins that play important yet distinct roles in the RNAi pathway. □

Methods

Drosophila cell culture and extract preparation

Drosophila S2 cells were cultured as described previously[29]. Extracts were prepared in two different ways. The first method was mainly used for chromatographic studies. Cells were washed with cold PBS and then lysed in buffer 5A (10 mM HEPES pH 7.0, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.75 mM DTT, 0.2 mM PMSF, protease inhibitors (Roche)) for 30 min and dounce homogenized. Nuclei were spun down, and the supernatant was saved as cytoplasmic extract. The nuclei were resuspended in buffer 5B (10 mM Tris pH 8.0, 500 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.75 mM DTT, 0.2 mM PMSF, protease inhibitors (Roche)) and extracted for 40 min on ice. The extracted nuclei were spun down and the supernatant saved as the nuclear fraction. Nuclear and cytoplasmic fractions were combined and spun at 20,000 *g* for 30 min (S10). The combined extract was used in most experiments. The second method was principally for immunoprecipitations and involved lysis of cells in buffer DmLB10 (25 mM Tris pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 0.2 mM PMSF, protease inhibitors (Roche)) and a 20,000 *g* 30 min spin.

Immunofluorescence

A modification of the protocol in ref. 30 was used. S2 cells were grown on slides coated with Concanavalin A (Sigma).

Pri-miRNA processing assay

DNA templates for transcription of Drosha substrates were reverse transcribed with the Superscript II Kit (Invitrogen) from total RNA prepared with Trizol (Invitrogen) using primers that flank the pre-miRNAs at least 50 bases. Primer sequences as follows: mir30a fwd and rev (see ref. 1); mir2a-1 T7 fwd: 5′-TAATACGACTCACTTAGGGATTCTGAA CTCCCGCAGTCAAGTATCC-3′, mir2a-1 rev: 5′-GTGTGAATTATGTGGCGGGAGG TATT-3′; mir-bantam T7-fwd: 5′-TAATACGACTCACTTAGGGCGCTCAGATGCAGA TGTTGTTGAT-3′, mir-bantam rev: 5′-GATCGGTCGGCATAAGTTCAAAGC-3′. Pri-miRNAs were transcribed and uniformly labelled with ^{32}P (Perkin Elmer) using the Riboprobe Kit (Promega). 5′ and 3′ labelling of cold-transcribed (Megascript kit, Ambion) RNA was done using guanylyltransferase (Ambion) and T4 DNA ligase (Amersham), respectively. A typical pri-miRNA processing reaction contained 1 μl 10xD (100 mM Tris pH 8.0, 60 mM $MgCl_2$, 5 mM DTT), 1 μl Rnasin (Promega), 0.2 μl ^{32}P-labelled pri-miRNA, 5 μl extract, 2.8 μl H_2O. Reactions were allowed to proceed for 2 h at 30 °C.

Chromatography

AKTA FPLC was used for column chromatography. All the columns and media were purchased from Amersham Biosciences. Buffers for ion exchange chromatography: IEX'A': 10 mM Tris pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 4% glycerol, 0.75 mM DTT; IEX'B': 10 mM Tris pH 7.5, 1 M NaCl, 1.5 mM $MgCl_2$, 4% glycerol, 0.75 mM DTT; for hydrophobic interaction chromatography HIC'A': 10 mM Tris pH 7.5, 1 M NaCl, 1.5 mM $MgCl_2$, 4% glycerol, 0.75 mM DTT; HIC'B': 10 mM Tris pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 4% glycerol, 0.75 mM DTT. For size chromatography, buffer IEX'A' was used.

C. elegans lacZ reporter assays

pkIs2084 was generated by X-ray mediated integration of the extrachromosomal transgene of strain CT5[23]. Note that *pkIs2084* often drives lacZ expression in a few (4–5) seam cells in the head region in a wild-type background. Adult animals are scored positive for a defect in *let-7*-mediated silencing only if the staining extends towards the tail. As control animals, either wild-type or *rrf-3(pk1426)* mutant animals were used. Animals injected with dsRNA against a gene not related to miRNAs (*mut-7*) were also used as a control, with identical results. Animals carrying *pkIs2084* were crossed with animals carrying *drsh-1(tm0654)*, *dcr-1(s2795)* or *pash-1(pk2083)*. Progeny of injected animals and progeny of animals homozygous for the transgene and heterozygous for *tm0654, pk2083* or *s2795* were fixed and stained with X-gal. The genotype of stained animals was verified by PCR followed by restriction digest or sequencing. *pash-1(pk2083)* was obtained by target-selected mutagenesis in *C. elegans* (E. Cuppen, unpublished results), and introduces a *Psi*I site. *pk2083* changes the TTA codon for L527 into TAA, introducing a premature stop.

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The Microprocessor complex mediates the genesis of microRNAs

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MicroRNAs (miRNAs) are a growing family of small non-protein-coding regulatory genes that regulate the expression of homologous target-gene transcripts. They have been implicated in the control of cell death and proliferation in flies[1,2], haematopoietic lineage differentiation in mammals[3], neuronal patterning in nematodes[4] and leaf and flower development in plants[5–8]. miRNAs are processed by the RNA-mediated interference machinery. Drosha is an RNase III enzyme that was recently implicated in miRNA processing. Here we show that human Drosha is a component of two multi-protein complexes. The larger complex contains multiple classes of RNA-associated proteins including RNA helicases, proteins that bind double-stranded RNA, novel heterogeneous nuclear ribonucleoproteins and the Ewing's sarcoma family of proteins. The smaller complex is composed of Drosha and the double-stranded-RNA-binding protein, DGCR8, the product of a gene deleted in DiGeorge syndrome. *In vivo* knock-down and *in vitro* reconstitution studies revealed that both components of this smaller complex, termed Microprocessor, are necessary and sufficient in mediating the genesis of miRNAs from the primary miRNA transcript.

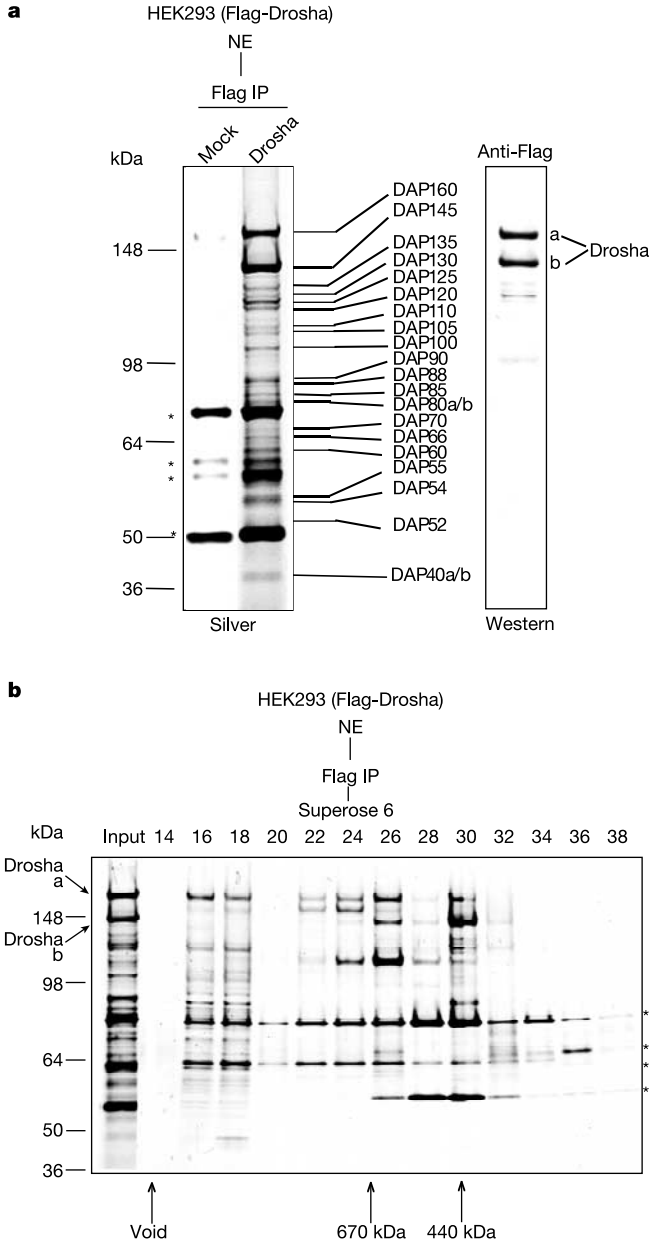

Figure 1 Isolation of Drosha-containing complexes. **a**, Fractions of the immunoaffinity eluate from M2 anti-Flag beads resolved by SDS–PAGE (4–12%). Flag-Drosha was revealed by silver staining and western blotting with anti-Flag antibodies. Molecular masses of marker proteins (left) and the polypeptide masses of associated subunits (right) are indicated. **b**, Silver staining of Superose 6 gel-filtration fractions. Top, fraction number; bottom, molecular mass markers. DAP, Drosha-associated proteins having different molecular masses; asterisks, contaminating polypeptides; IP, immunoprecipitation.