The Soluble And Polymeric Tubulins in the Ovary, Oocyte and Embryo of Zebrafish: Megadalton Soluble Tubulin Complexes and Gamma Tubulin

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Abstract

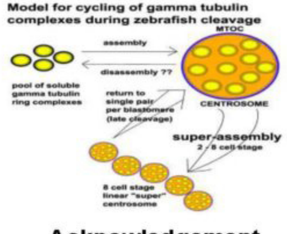
Tubulin dynamics, i.e., the interchange of polymeric and soluble forms in zebrafish oogenesis and embryogenesis, is important for microtubule (MT) cellular functions. The majority of α-tubulin in zebrafish oocytes was present in the soluble and not the polymer pool. Using differential centrifugation, gel chromatography and DM1A probed western blot, soluble α-tubulin was found to be associated with large molecular weight complexes (>2MDa) which were reduced by the blastula stage, with a concomitant decrease in soluble tubulin amount. Complexes (< 2MDa) then increased in the gastrula with an increase in soluble α-tubulin. Unlike MT fragments, the tubulin complexes were freeze-thaw resistant and stable in high salt. Two different anti-γ-tubulin monoclonal antibodies, GTU 88 and TU 30, revealed the existence of γ-tubulin in both zebrafish oocytes and embryos, which also decreased by the blastula stage and increased in the gastrula stage. Soluble α-tubulin and γ-tubulin of zebrafish ovaries, oocytes and embryos were co-localized in fractions from three different columns: Sephacryl-200, DEAE and Superose-6b. Ovarian soluble α-tubulin was co-immunoprecipitated with γ-tubulin. Immunofluorescence microscopy revealed discreet γ-tubulin foci in centrosomes and diffuse labeling in blastomere cytoplasm in early embryos. In 2-16 cell embryos, clusters of centrosomes were seen, sometimes forming linear arrays. Injection of anti-sense oligos to γ-tubulin resulted in A-P axis defects. In situ hybridization with γ-tubulin oligo revealed diffuse label in oocytes, with a marked localization to the blastodisc upon maturation. Ovary and eggs showed similar patterns of tubulin gene product expression while differing from day 4 larva using microarray. These findings, together with recent work on γ-tubulin ring complexes in other species, suggest that γ-tubulin protein complexes may be involved in regulating tubulin dynamics during zebrafish oogenesis and embryogenesis.

Introduction

Zebrafish (*Danio rerio*), a small tropical freshwater teleost, has emerged as a model for cell and developmental biology because of its high fecundity, short generation time and rapid development of the externally fertilized and translucent embryos (Driever *et al*., 1994). Microtubules (MTs) and tubulin dynamics, i.e., the interchange of polymeric and soluble forms, are important in a variety of cell functions during oogenesis and embryogenesis in zebrafish. Besides their well-known role in mitosis, MTs are required for epiboly (Solnica-Krezel and Driever, 1994; Strahle and Jesuthasan, 1993), furrow formation (Pelegri *et al.*, 1999), and the cohesion of post-cytokinesis blastomeres in zebrafish (Jesuthasan, 1998). As transport lines for regulatory substances and maternal mRNAs, MTs are also required for transportation of dorsal determinants, axis determination and establishment of embryonic polarity in zebrafish (Jesuthasan and Strahle, 1997). Therefore, MT dynamics and organization must be regulated both temporally and spatially (Becker and Gard, 2000).

The classic paradigm for tubulin dynamics is based on "dynamic equilibrium" between polymer and soluble heterodimer tubulin pools. Thus, as soluble tubulin increases in concentration, polymer assembly should ensue. However, preliminary studies revealed that the majority of α-tubulin in zebrafish oocytes was present in the soluble and not in the polymer pool (Liu and Lessman, 2001); this predominance of soluble tubulin was also found in *Xenopus* (Pestell, 1975), *Drosophila* (Raff *et al*., 1975), Sea urchin (Raff and Kaumeyer, 1973) and *Rana pipiens* oocytes (Wang and Lessman, 1997). This finding is unusual because in most other cell types, the majority of α-tubulin is in MTs form (polymer). Why does this soluble tubulin persist without spontaneous assembly into MTs? Does this soluble tubulin still dominate in zebrafish embryos? We hypothesized that soluble tubulin was accumulated and stored in an "oligomeric" form that was composed of large molecular weight complexes and did not readily assemble into MTs during zebrafish oogenesis. It is further hypothesized that these large tubulin complexes, especially in fully-grown oocytes, would be sub-divided into many smaller complexes and incorporated into embryonic MTs networks in future embryogenesis.

In animal cells, microtubules are nucleated by the centrosome, which consists of a centriole pair surrounded by pericentriolar material (PCM). The centrosomal protein γ-tubulin seems to be a key factor in microtubule nucleation (Pereira and Schiebel, 1997; Wiese and Zheng, 1999). So far it is known to function as a multi-protein complex, called the γ-tubulin ring complex (γTuRC), which composes the microtubule-organizing center (MTOC). The data to date indicate that soluble γ-tubulin exists as large protein complexes with α- and β-tubulin in *X. laevis*, sheep (Wiese and Zheng, 1999) and *Rana pipiens* (Lessman and Kim, 2001). The above results showed that the MDa tubulin complexes contain α-, β- and γ-tubulin as well as other proteins. Besides its important role in nucleating microtubules, γ-tubulin is also necessary in many other cellular functions. The present study was undertaken to provide basic biochemical, molecular and physiological data on tubulins, including γ-tubulin, in zebrafish oocytes and embryos. And to provide a foundation for future work on the tubulin cytoskeleton in this important model organism.



Acknowledgement

We thank Drs. Thomas Sutter and Shirlean Goodwin for their help in total RNA isolation and microarray assay. We Thank Dr. P. Draber for a gift of TU-30; the University of Memphis Graduate School and the Department of Biology supported this research.

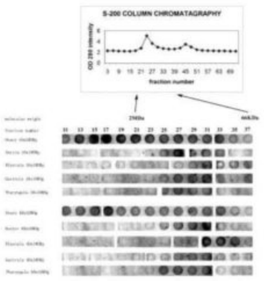


Figure 1: Comparison of immuno-dot blots (probed with DM1A) of supernatant preparations of different developmental stages of oogenesis and embryogenesis run on an S-200 Sephacryl column.
Top panel: OD_{280} profile of zebrafish protein from oocyte or embryo supernatants eluted from an S-200 Sephacryl column. The standards Dextran blue and BSA eluted at the 21st fraction (2 MDa) and 37th fraction (66 KDa), respectively. **Lower panel**: Comparison of immuno-dot blots (probed with DM1A) of column fractions of supernatant from different developmental stages of oogenesis and embryogenesis eluted from one S-200 Sephacryl column. The molecular weight standards used to calibrate the column are indicated above. Odd numbered fractions (100μl) were spotted; while even numbered fractions were used for Western blots.

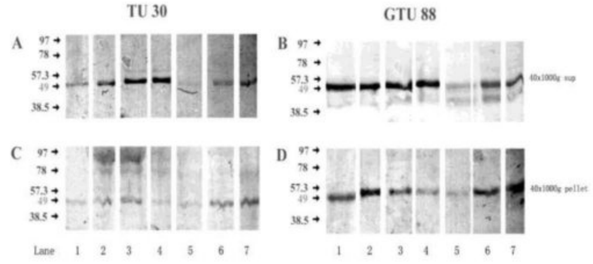


Figure 2: Western blot of zebrafish oocyte or embryo extracts at the indicated developmental stages probed by anti γ-tubulin antibodies (GTU 88 or TU 30).
Panel A: original 40x1000g supernatant probed by TU 30. **Panel B:** original 40x1000g supernatant probed by GTU 88. **Panel C:** original 40x1000g pellet probed by TU 30. **Panel D:** original 40x1000g pellet probed by GTU 88.
Lane 1) fully-grown oocyte. 2) 1-4 cell, 3) 8-64 cell, 4) 64-128 cell, 5) mid-blastula (>1000 cells), 6) gastrula, 7) pharyngula.

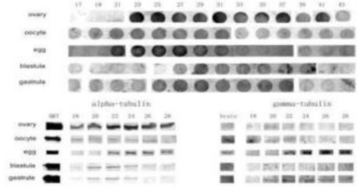


Figure 3: Comparison of immuno-dot blots (probed with GTU 88) of 16x1000g supernatant preparations of different developmental stages of oogenesis and embryogenesis run on an S-200 Sephacryl column. Odd numbered fractions (100μl) were spotted; while even numbered fractions were used for Western blots (lower panel, probed by DM1A and GTU 88). The standards Dextran blue and BSA eluted at the 21st fraction (2 MDa) and 37th fraction (66 KDa), respectively.
Lower panel: Western blot analysis (probed with DM1A and GTU 88) of supernatant preparations of different developmental stages of oogenesis and embryogenesis run on an S-200 Sephacryl column. BBT: Bovine brain tubulin standard. 16x1000g S-200 column fractions (from 18 to 28) are indicated above each lane

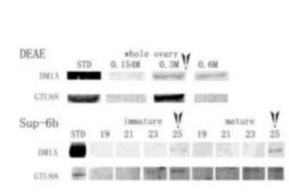


Figure 4: Western blots analysis (probed with DM1A and GTU 88) of column fractions of supernatant extractions from zebrafish ovaries, fully-grown (immature) and mature oocytes after 16x1000g centrifugation and elution from either DEAE or Superose-6b column. Zebrafish brain 16x1000g supernatant extract was used to be standard (std). Arrows indicated the co-elution of α-tubulin and γ-tubulin from each column.
DEAE: Three different NaCl-50mM Tris buffer (0.154M, 0.3M and 0.6M, pH 7.4) which eluted the ovarian supernatant extractions were indicated above each pooled and concentrated peak fractions.
Sup-6b: Fraction numbers (from 19-25) indicated each concentrated fraction for both immature and mature oocyte sections.

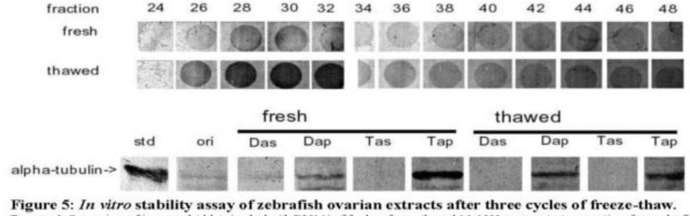


Figure 5: *In vitro* stability assay of zebrafish ovarian extracts after three cycles of freeze-thaw.
Top panel: Comparison of immuno-dot blots (probed with DM1A) of fresh or freeze-thawed 16x1000g supernatant preparations from zebrafish ovaries run on an S-200 Sephacryl column. Each fraction number (from 24 to 48) indicated corresponding individual fraction.
Lower panel: Western blots analysis (probed with DM1A) of microtubule *in vitro* assembly assay aided by taxol (T) with fresh or freeze-thawed 16x1000g supernatant preparations from zebrafish ovaries. Zebrafish brain extracts was used as standard (std). "ori" means fresh original zebrafish 16x1000g supernatant. DMSO (D) was used as a vehicle control. "as" means supernatant after assembly. "ap" means pellet after assembly.

Table 1: Comparison of different gamma-tubulin-antisense injection effects in early zebrafish embryos using ratios of the number of abnormal (axial defects) embryos to the total live embryos injected after 24 hrs. * indicates significant difference in each oligo-nucleotide injection group compared to vehicle control ($P < 0.02$) using z-test proportion comparison assay.

Treatment	Abnormal/ total alive	Percent (%)
Control	6/182	3.3
Oligo 1		
125 pg/nl	48/179	27*
500 pg/nl	10/42	24*
Oligo 1'		
125 pg/nl	33/253	13*
500 pg/nl	9/68	13*
Oligo 2		
125 pg/nl	13/105	12*
500 pg/nl	4/16	25*
Oligo 2'		
125 pg/nl	15/122	12*
500 pg/nl	3/15	20*

Table 2: Microarray data for wild-type zebrafish ovary, mature egg and 4 day larval tubulins and related mRNAs. Presented as relative abundance.

day 4 larva	egg	ovary	Target Description	probe	Accession number
477	783	609	tubulin, gamma 1	Dr.11201.1	gb:BC045486.1
121	370	443	gamma-tubulin complex protein 2 gamma-tubulin interacting protein (yeast SPC98 homolog)	Dr.3637.1	gb:BG302753
	620	241		Dr.15107.1	gb:BM182344
9012			tubulin, alpha 1	Dr.11310.1	gb:BC042319.1
4109	371	770	tubulin, alpha 1	Dr.11310.2	gb:AF029250.1
2956	514	1329	tubulin, alpha 1	Dr.11310.2	gb:AF029250.1
6633			tubulin, alpha 1	Dr.11310.3	gb:BG306212
2386			tubulin, alpha 1	Dr.11310.3	gb:BG306212
14460	2207	2310	alpha-tubulin	Dr.11310.4	gb:BG615090
6783	3631	4407	tubulin, alpha 8 like	Dr.20010.2	gb:AW115602
1028			tubulin, alpha 8 like 3	Dr.20214.1	gb:AI793708
214	15701	12510	tubulin, alpha 4 like	Dr.23436.1	gb:BC046889.1
307	199	179	tubulin, alpha 2 isoform 1	Dr.25456.1	gb:BI673876
	1556	1440	Tubulin alpha-1 chain, brain-specific	Dr.25680.1	gb:AL716150
1866			tubulin, alpha 2	Dr.26381.1	gb:BI866299
26200	13743	11188	tubulin, alpha 8 like 4	Dr.664.1	gb:BC045847.1
			DEAD (Asp-Glu-Ala-Asp) box polypeptide		
3164	3913	3094	5	Dr.664.2	gb:AA658691
41077	3018	2267	tubulin, alpha 1	Dr.664.3	gb:BI326418
4252	142	196	tubulin, alpha 8 like 2	Dr.7506.1	gb:BE017692
1220	10364	9885	Tubulin beta-5 chain	Dr.24758.1	gb:BQ078442
7641			Tubulin beta-5 chain	Dr.24758.2	gb:BQ078442
3155	78	54	Tubulin beta-2 chain	Dr.24902.1	gb:AL717250
1587	324	105	tubulin, beta 5	Dr.4416.1	gb:AF528096.1
11886	10441	7670	tubulin, beta, 2	Dr.5605.1	gb:AI397104
14653	11396	8078	tubulin, beta, 2	Dr.5605.2	gb:BM141612
14362	5227	5065	tubulin beta mRNA	Dr.5605.3	gb:AI477242
13503	5424	4897	tubulin beta	Dr.5605.3	gb:AI477242
14108	431	554	tubulin beta	Dr.5605.4	gb:BQ263917
489	135	92	beta-tubulin	Dr.6173.1	gb:AW077444
2445	1978	1913	tubulin cofactor a beta-tubulin cofactor C;	Dr.1163.1	gb:BC046032.1
485	1852	1873	tubulin-specific chaperone c	Dr.16676.1	gb:BI980285

Figure 6: Western blots analysis (probed with DM1A) of stability assay of zebrafish ovarian extracts treated with different concentration of NaCl or pH and run on an S-200 Sephacryl column. Zebrafish brain extracts were used as a tubulin standard (STD). Fraction numbers (from 20 to 34) are indicated at the top of panel. Different treatments are listed to the left of each panel.

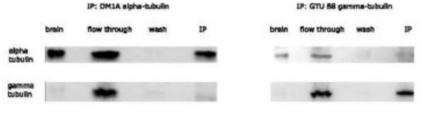


Figure 7: Co-immunoprecipitation assay of α-tubulin and γ-tubulin from 16x1000g zebrafish ovarian extracts using DM1A or GTU 88-conjugated protein G beads. **Western blots probed (label at left) for α-tubulin (DM1A) or γ-tubulin (GTU-88) followed by Tru-Blot HRP-goat anti-mouse (detects native IgG) with chemiluminescent detection.**

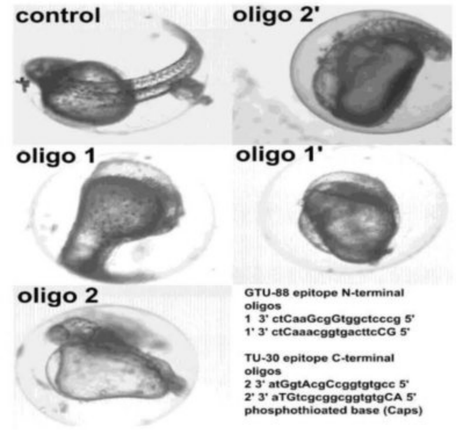


Figure 8. Representative phenotypes of anterior-posterior axis defects 24hr post-injection with γ-tubulin antisense oligonucleotides.
Oligonucleotides 1 & 2 were designed from human γ-tubulin gene sequence, while 1' & 2' were designed from the zebrafish gene. Control received vehicle only. Injectate volume = 2 nl, 2 - 8 cell stage.

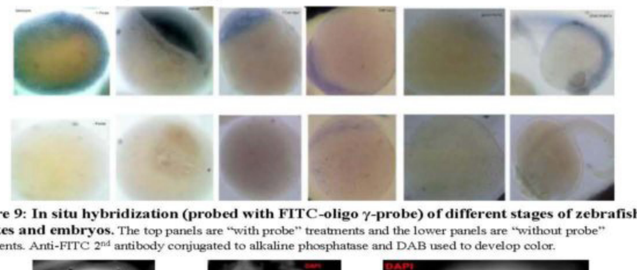


Figure 9: In situ hybridization (probed with FITC-oligo γ-probe) of different stages of zebrafish oocytes and embryos. The top panels are "with probe" treatments and the lower panels are "without probe" treatments. Anti-FITC 2nd antibody conjugated to alkaline phosphatase and DAB used to develop color.

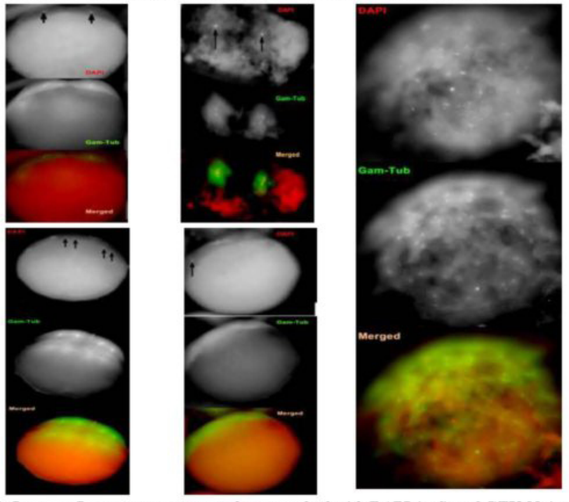


Figure 10: Immunofluorescence assay: embryos probed with DAPI (red) and GTU 88 (green).
Arrows indicate nuclei in 2 – 8 cell embryos (left & middle panels). Lower left, 8 cell embryo with linear array of centrosomes. At right, gastrula has more numerous and smaller centrosomes.

Conclusions

1. Soluble α-tubulin was found to be associated with large molecular weight complexes (>2MDa) which were reduced by the blastula stage, with a concomitant decrease in soluble tubulin amount. Complexes (< 2MDa) then increased in the gastrula with an increase in soluble α-tubulin.
2. Both soluble and polymerized γ-tubulin existed in zebrafish oocytes and embryos, which also decreased by the blastula stage and increased in the gastrula stage.
3. Unlike MT fragments, the tubulin complexes were freeze-thaw resistant and stable in high salt.
4. Soluble α-tubulin and γ-tubulin of zebrafish ovaries, oocytes and embryos were co-localized in fractions from three different columns: Sephacryl-200, DEAE and Superose-6b. Ovarian soluble α-tubulin was co-immunoprecipitated with γ-tubulin.
5. Injection of anti-sense oligos to γ-tubulin resulted in A-P axis defects.
6. In situ hybridization with γ-tubulin oligo revealed diffuse label in oocytes, with a marked localization to the blastodisc upon maturation.
7. Immunofluorescence microscopy revealed discreet γ-tubulin foci in centrosomes and diffuse labeling in blastomere cytoplasm in early embryos.
8. Ovary and eggs showed similar patterns of tubulin gene product expression while differing from day 4 larva using microarray.